

ENDOMETRIOTIC TISSUE AND UTERINE ENDOMETRIUM

Comparative studies on morphology
and steroid binding

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Title and subtitle ENDOMETRIOTIC TISSUE AND UTERINE ENDOMETRIUM. Comparative studies on morphology and steroid binding.		
Abstract Endometriotic tissue was compared to uterine endometrium from the same woman. A cyclic menstrual phase pattern was found histologically in the endometriotic tissue in 82% of the cases and in 65% the cycle phase was congruent to the endometrial phase. The anatomical localization of the endometriotic lesion did not influence the phase pattern. Differences in phase pattern might depend on differences in steroid receptor content in the two types of tissue. Estrogen (ER) and progesterone (PR) receptors were therefore assayed biochemically. ER was measurable in only 8/20 and PR in only 2/9 endometriotic samples, and the amount was always lower than that in endometrium. However, biochemical assays are less suitable for small tissue samples. Therefore, the specificity of a histochemical method for demonstration of steroid binding using E ₂ -BSA-FITC and P-BSA-TMRITC was evaluated and found to be acceptable. Both fluorochrome labeled steroids bound to epithelial and, to a less degree, to stromal cells in the endometrium and the distribution of the binding changed during the menstrual cycle. The binding extent in the epithelial component of endometriotic tissue did not obviously differ from that in the endometrium although no apparent cyclic changes were found. After transplantation into nude mice treated with exogenous hormones similar histological patterns were found in the two tissue types. Fibrous tissue often surrounds and invades endometriotic lesions in humans and a similar fibrous reaction was seen in the endometriotic as well as the endometrial grafts. The fibrous ingrowth influenced the appearance of the glands. After a few weeks in nude mice the two tissue types had assumed the same appearance, similar to human endometriotic tissue, and the origin of the graft could no longer be identified. It was concluded that endometriotic tissue contains binding sites for estrogen and progesterone, that endometriotic tissue and uterine endometrium transplanted to nude mice respond similarly to hormonal stimuli and that the histological and functional differences between endometriotic tissue and uterine endometrium, at least partly, may depend on local factors in the surrounding tissue.		
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ENDOMETRIOTIC TISSUE AND UTERINE ENDOMETRIUM
Comparative studies on morphology and steroid binding

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Comparative studies on morphology and steroid binding

by

Agneta Bergqvist

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The history of endometriosis is one of the most interesting stories in medicine.

J.H. Ridley, 1968

"If thou examinest a woman who has pains in one side of her hypogastric region, then thou shalt say thereto: 'It is (due to) disorder of her menstruations'. Now after it has appeared then thou shalt prepare for her; crushed onion, subt (sic) and sawdust of pine. The hypogastric region is bandaged herewith."

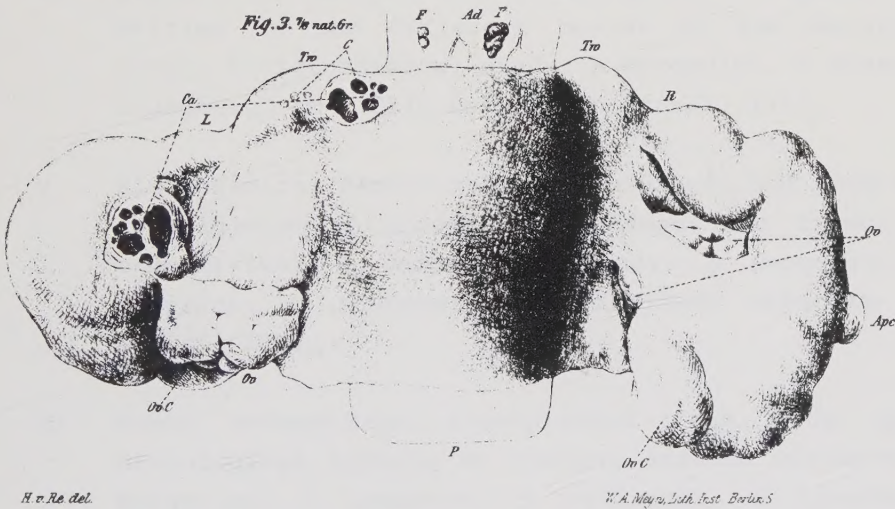
From 1600 BC

Adenomyoma
 Adenomyosis
 Chocolate cyst
 Hemorrhagic perforating cyst
 Menstruating cyst
 Endometriosis
 Endometrioma
 Adenoma endometrioides ovarii
 Internal endometriosis
 External endometriosis
 Cystosarcoma adenoides uterinum
 Sarcoma adenoides uterinum polyposum
 Fibroadenoma cysticus et polyposum
 Cystadenomyoma
 Adenomyoma uteri diffusum benignum
 Hysteroadenosis metastatica
 Endometrioiden heterotopien
 Uterine endometriosis
 Ectopic endometrium
 Endometrial endometriosis
 Endometrial intra-uterine endometriosis
 Parietal uterine endometriosis
 Mixed endometriosis
 Stromatous endometriosis
 Mixed endometriomata
 Stromatous endometriomata
 Parietal endometriosis
 Mixed parietal endometriosis
 Interstitial endometriosis
 Chronic uterine stromatous endometriosis
 Chronic uterine stromatous endometriomata
 Ovarian stromatous endometriosis
 Cervical endometriosis
 Pelvic endometriosis
 Internal uterine endometriosis
 External uterine endometriosis
 Von Recklinghausen's disease
 Adenomyomatosis
 Adenometritis
 Adenomyositis
 Adenomyometritis
 Endometrio fibroma
 Endometrio fibromyoma
 Adenofibromatosis heterotopica
 Chocolate cyst

Hysteroadenosis metastatica
 Adenoma of endometrial type
 Implantation adenoma
 Ovarian haematomas of endometrial type
 Pelvic adenomas of endometrial type
 Adenofibromyoma cysticum sarcomatodes carcinomatosum
 Endometriosis müllerianosis
 Müllerianoma
 Endometriomyoma
 Cystoma endometriodes ovarii

The term endometriosis was coined by Sampson 1921

"What's in a name? that which we call a rose
 By any other name would smell as sweet"
 W. Shakespeare



Rokitansky C, 1860

Tag vara på livets möjligheter

This thesis is based on the following papers, referred to in the text by their Roman numerals:

- I Human endometrium and endometriotic tissue obtained simultaneously: A comparative histological study. A Bergqvist, O Ljungberg, E Myhre. Internat J Gynecol Pathol 3:135-145, 1984.
- II Estrogen and progesterone cytosol receptor concentrations in endometriotic tissue and intrauterine endometrium. A Bergqvist, G Rannevik, J Thorell. Acta Obstet Gynecol Scand Suppl 101:53-58, 1981.
- III Histochemical localization of estrogen and progesterone receptors: Evaluation of a method. A Bergqvist, K Carlström, O Ljungberg. J Histochem Cytochem 32:493-500, 1984.
- IV Binding of estrogen and progesterone to human endometrium in the different phases of the menstrual cycle. A histochemical study. A Bergqvist, R Ekman, O Ljungberg. Am J Clin Pathol (in press), 1984.
- V Histochemical demonstration of estrogen and progesterone binding in endometriotic tissue and in uterine endometrium: A comparative study. A Bergqvist, S Jeppsson, O Ljungberg. J Histochem Cytochem (in press), 1984.
- VI Human endometrium transplanted into nude mice. Histological effects of various steroid hormones. A Bergqvist, S Jeppsson, S Kullander, O Ljungberg. Submitted for publication 1984.
- VII Human uterine endometrium and endometriotic tissue transplanted into nude mice. Morphological effects of various steroid hormones. A Bergqvist, S Jeppsson, S Kullander, O Ljungberg. Submitted for publication 1984.

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LIST OF ABBREVIATIONS

AFP	Alphafetoprotein
AR	Androgen receptor
BSA	Bovine serum albumin
CBG	Corticosteroid binding globulin
CR	Corticoid receptor
Danazol	17 α -Pregn-4-en-20-yno[2,3-d]-isoxazol-17-ol
DCC	Dextran-coated charcoal
DES	Diethylstilbestrol; trans 3,4-bis(p-hydroxy-phenyl)-3-hexene
5 α DHT	5 α -dihydrotestosterone
DNA	Deoxyribonucleic acid
E ₂	Estradiol-17 β
ERc	Cytoplasmic estrogen receptor
FITC	Fluorescein isothiocyanate
fmol	femtomoles
H	Hormone
IFPAG	Isoelectric focusing in polyacrylamide gel
i.p.	Intraperitoneally
mRNA	Messenger ribonucleic acid
MPA	Medroxyprogesterone acetate
ORG2058	16 α -ethyl-21-hydroxy-19-nor-4-pregnene-3,20-dione
P	Progesterone
PEP	Polyestradiol phosphate
PRc	Cytoplasmic progesterone receptor
R1881	Methyltrienolone; 17 β -hydroxy-17 α -methyl-estra-4,9,11-triene-3-one
R5020	Promestone; 17 α ,21-dimethyl-19-nor-pregna-4,9-diene-3,20-dione
Rc	Cytoplasmic receptor
Rn	Nuclear receptor
RNA	Ribonucleic acid
RTF	Receptor transforming factor
S	Svedberg unit
s.c.	Subcutaneously
SHBG	Sex hormone binding globulin
Tamoxifen	Trans-1-(4 β -diethylaminoethoxyphenyl)-1,2-diphenyl but-1-ene
TMRITC	Tetramethylrhodamine isothiocyanate

INTRODUCTION

Endometriosis is defined as the occurrence outside the uterine cavity of areas of glandular structures surrounded by specific stroma, histologically and functionally resembling uterine endometrium. Although they may be found in any organ in the body, the lesions predominantly occur in the pelvic region, involving the ovaries, broad ligaments, rectovaginal septum, uterosacral ligaments, uterovesical pouch, the surface of uterus and oviducts (Jeffcoate, 1967). Most of endometriotic lesions are small, only a millimeter or two in diameter, but sometimes, especially in the ovaries, they may develop into blood filled cysts, endometriomas. Endometriotic tissue may invade and destroy surrounding structures, which is unique among benign adult tissues. The lesions are surrounded by fibrotic tissue, which often gives rise to firm adhesions. This reaction may sometimes be very pronounced, and a conglomerate mass of firm adhesions and endometriomata, together with the pelvic organs, may create a pelvic block and seal off the cul-de-sac.

Although the natural course of endometriosis is essentially unknown, the disease is usually regarded as progressive, even if signs of spontaneous healing are sometimes found.

Endometriosis is a common disease in women of fertile age, and most prevalent between the ages of 30 and 40 (Richter, 1973; Ranney, 1978; Riedel & Semm, 1980), although the condition is not uncommon in younger women (Schiffrin et al, 1973). As the disease is often asymptomatic and remains undiagnosed, its true incidence is not known (Tauber, 1965), but among women exposed to gynecological laparotomy or laparoscopy, its frequency has been reported to be 10-50% (Meigs, 1953; Ranney, 1978; Riedel & Semm, 1980). The disease can be extremely disabling, the common-

est symptoms being pain (acquired dysmenorrhea, deep dyspareunia, painful defecation, diffuse pain in the pelvic region or lower back), menstrual abnormalities and infertility (Counsellor et al, 1951; Riedel & Semm, 1980; Muse & Wilson, 1982).

Although the etiology of endometriosis is obscure, cyclic ovarian estrogen-progesterone production is regarded as a prerequisite for its development (Ranney, 1978). However, endometriosis has been reported in anovulatory women (Soules et al, 1976). Furthermore, endometriosis has also been reported in a woman with Turner's syndrome, treated cyclically with estrogen and progesterone (Binns & Banerjee, 1983), as well as in three men with prostatic cancer, treated with estrogen (Schrodt et al, 1980). Even if surgery most occasionally be resorted to (Ranney, 1971; Pratt & Williams, 1980), the therapeutic approach increasingly adopted over the past 40 years has been hormonally induced anovulation. The aim of pharmacological treatment is to achieve atrophy in endometriotic lesions similar as in endometrium. However, about 10-20% of the patients do not respond to hormonal treatment (Kistner, 1966; Hammond & Haney, 1978; Andrews, 1980).

Even if the lesions have been regarded as ectopic endometrium, only few comparative studies have been performed on the histology and response to hormonal stimuli of the two types of tissue. Parallel advances in knowledge of uterine physiology and biochemistry have brought about a reinterpretation of the fine structural cyclic changes occurring in the human endometrium and in endometriotic tissue.

The main purposes of the present work have been:

to make a detailed histological comparison of endometriotic tissue and uterine endometrium in different phases of the menstrual cycle.

to study some of the prerequisites for hormonal response - i.e., whether the amount and localization of specific steroid binding proteins are the same both in endometriotic tissue and in uterine endometrium.

to study the histological response in endometriotic tissue and uterine endometrium transplanted to similar foreign milieu and exposed to exogenous hormones.

LITERATURE REVIEW

THEORIES OF THE PATHOGENESIS OF ENDOMETRIOSIS

Since ectopic endometrial structures were first reported by Rokitansky (1860), much has been written on ectopic endometrium (for a review see Gardner et al, 1953). Even if many theories have been postulated through the years (for review see Ranney, 1948), the pathogenesis of endometriosis is still largely unknown.

One of the most widely accepted theories is that of implantation, proposed by Sampson, who suggested that viable fragments of uterine mucosa are transported with menstrual blood in retrograde fashion through the oviducts, and implanted on pelvic structures (Sampson, 1922).

Both lymphogenous dissemination (Sampson, 1922; Halban, 1924; Javert, 1952), and hematogenous dissemination (Sampson, 1927; Abdel-Shahid et al, 1974), have been put forward to explain lesions elsewhere in the body.

Endometriotic lesions in laparotomy scars after hysterotomies have given rise to the suggestion of mechanical implantation (von Franqué, 1916). Other mechanical factors such as curettage, insufflation and cervical obstruction have also been suspected of inducing endometriosis by implantation.

An in situ development of ectopic endometrium from local cells has been considered yet another possible explanation. Iwanoff (1898) postulated the coelomic metaplasia theory, which was later supported by Gruenwald's fundamental embryological studies (1942). Adult derivatives of the embryonic coelomic cells (including peritoneum and pleura) may retain the potential to undergo metaplastic changes and form tissue that is histologically and functionally similar to endometrial tissue. The coelomic

metaplasia theory was championed by Meyer (1927), who suggested that the metaplasia is induced by inflammatory stimuli, and by Novak (1926), who proposed that endometriotic tissue has its origin in the ovaries, the cells being sensitized by hormones secreted from the ovaries.

Other authors found indications of an imbalance in ovarian steroid production. Jeffcoate & Potter (1934) believed that an estrogenic overstimulation caused the growth of ectopic endometrium, and in some other reports (Brosens et al, 1978; Hargrove & Abraham, 1980) indications of an inadequate corpus luteum function have been reported.

Moreover, indications of involved immunological reactions have been published. Dmowski et al (1981) suggested that translocated endometrial cells may implant only in women with specific alterations in their cell-mediated immunity.

MACROSCOPICAL AND HISTOLOGICAL STUDIES ON HUMAN ENDO-METRIOTIC TISSUE AND UTERINE ENDOMETRIUM

During different phases of the menstrual cycle, Ottow (1929) made repeated cystoscopic examinations of two foci of endometriosis in the bladder, and observed changes that reflected those of the menstrual flow from the endometrium. In certain cases of endometriosis in the inguinal region, Lissowetzky & Bujko (1932) noted simultaneous changes in endometriotic and uterine endometrial tissue. Hoffmann et al (1953), investigating endometriosis located in the mucous membrane of the vagina, observed that blood congestion had already occurred in the lesions 84 hours before the onset of the menstrual flow, after which the lesions soon began to diminish, and within 48 hours the blood congestion had disappeared completely. Thus macroscopical studies have indicated that endometriotic structures and uterine endometrium react in a similar way.



Human uterine endometrium has been extensively studied histologically, and the changes through the menstrual cycle are known in detail (Noyes et al, 1950; Wynn, 1977; Ferenczy et al, 1979).

In several studies, the morphology of endometriotic tissue has been reported to be identical to that of eutopic endometrium and the histological menstrual phase pattern has been found to parallel that of the normal uterine mucosa (Jeffcoate & Potter, 1934; Roddick et al, 1960; Nieminen, 1962). Chydenius (1933) reported two cases of very extensive endometriotic lesions in the vaginal fornix from which he obtained repeated samples for histological examination, comparison being made with simultaneously obtained uterine endometrium; he recognized distinct parallel cyclic changes in the two tissues.

In many other reports, however, deviations from the uterine cycle phase pattern has been noted in endometriotic structures: a delay in the cycle phase (Schweppe et al, 1984); a cycle phase in advance of that in the uterus (Roddick et al, 1960); or an inconsistent correlation to the endometrial cycle phase (Scott & Wharton, 1955). Novak & de Lima (1948) and Novak & Hoge (1958) found, that in some cases the endometriotic glandular pattern corresponded well to the histological cycle phase in the uterine endometrium, but that in others the glands were quiescent, and the authors suggested a defect response to progesterone. Moreover, they stressed the occurrence of bleeding in the endometriotic structures even during intermenstrual periods. Some other authors (Ranney, 1978; Schweppe et al, 1984) found that different glands in the same sample, and sometimes different cells in the same gland, respond to hormonal stimuli to varying degrees. Moreover, the occurrence of epithelial atypia in the ectopic lesions has been stressed by Sampson (1927) and Czernobilsky & Morris (1979).

Several suggestions have been put forward to account for the varying reactions to hormonal stimuli: differences in anatomical localization (Dallenbach-Hellweg & Grumbrecht, 1975); differences in local milieu (Chydenius, 1933; Nieminen, 1962); variations in the stages of growth (Sampson, 1921); disturbed nutrition and hormonal effect as a result of the surrounding fibrosis (Ranney, 1978); and that different lesions represent different stages of a transition between either germinal or follicular epithelium and genuine endometrial epithelium (Novak, 1926). The importance of the stroma was emphasized by Cullen (1896). Seitz (1932) proposed that variations in growth activity found in different glands are dependent on the degree of pressure obtaining in the lesions.

In an ultrastructural study of endometriotic tissue and uterine endometrium obtained simultaneously from the same woman, Schweppe et al (1981) reported that endometriotic tissue usually, but not always, exhibits the characteristics of uterine endometrium in the same phase of the cycle, although endometriotic implants often exhibit features that represent an incomplete response to the prevalent hormonal milieu, both in the proliferative and the secretory phases.

Thus the morphology of endometriotic structures and the changes during the menstrual cycle have been extensively studied, though the results are controversial. However, no detailed light microscopical comparison has been published of the histological pattern in endometriotic tissue and uterine endometrium obtained simultaneously from several women.

BIOCHEMISTRY OF ESTROGEN (ER) AND PROGESTERONE (PR) RECEPTORS; RECEPTOR ASSAYS

According to current theory on steroid hormone action, the

presence of an intracellular receptor is considered as a prerequisite for hormonal response. The existence of specific estrogen-binding proteins in estrogen target tissues was first described by Jensen & Jacobson 1962, and a quantitative assay of estrogen receptors was first performed by Toft & Gorski 1966. Since then, steroid receptors have been extensively studied (for review and references see Gorski & Gannon, 1976; Clark & Peck, 1977; Edwards et al, 1979; Tseng, 1979).

The traditional steroid receptors are acid proteins, which are characterized by high stereo-specificity of the binding, high binding affinity to the ligand (dissociation constant of the steroid-receptor complex $\approx 10^{-9} - 10^{-10}$ M), limited binding capacity at near physiological concentrations of the ligand, and they are only found in tissues sensitive to steroid hormones (Clark & Peck, 1977). The receptor sediment as an 7-8S protein on sucrose gradient centrifugation. Also a 4S component is obtained, the function of which is uncertain.

Even if some indications of an active transport of the steroids over the cell membrane have been reported (Milgrom et al, 1973), it is widely accepted that the unbound fractions of steroid hormones diffuse through cell membranes (Clark & Peck, 1977; Müller & Wotiz, 1979) and bind non-covalently to receptor proteins in the cytoplasm (Fig. 1). After hormone binding, the receptor is activated, and translocated into the nucleus, where it attaches to specific chromatin acceptor sites. Gene transcription follows, resulting in synthesis, or inhibition of synthesis, of specific proteins, which constitute the characteristic end-product of the action of steroids on cells.

Both ER and PR synthesis is stimulated by estrogen and inhibited by progesterone. Two types of specific estrogen

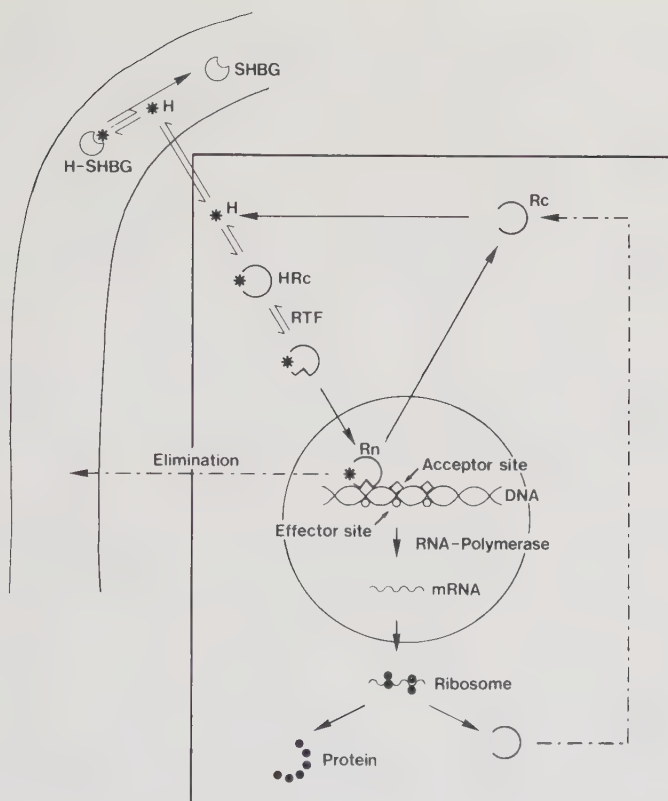


FIG 1

binding proteins have been described, type I and type II (Eriksson et al, 1978). Type I is the conventional cytoplasmic 8S receptor protein with very high specificity and affinity to the hormone. After estrogen stimulation type I receptor sites are translocated from the cytoplasm to the nucleus. Type II is a 4S macromolecule and is usually present in conjunction with type I. Their respective concentrations are strongly correlated (Clark et al, 1978; Eriksson et al, 1980; Panko et al, 1981). Even type II is a specific estrogen binding site which has a lower affinity and higher capacity than do the type I ER. Type II estrogen binding protein has been found predominantly in

estrogen target tissue, and may be of physiological significance, as their occurrence is related to uterine growth response to estrogenic stimulation. Estrogen stimulation of true uterine growth seems to require long term nuclear retention of type I sites and sustained elevation in type II sites level (Markaverich & Clark, 1979). However, unlike type I, the cytoplasmic type II site have not been found to undergo nuclear translocation (Clark et al, 1978; Eriksson et al, 1980), but a nuclear type II estrogen binding site exists as well, the level of which increases after estrogen administration (Eriksson et al, 1978; Markaverich & Clark, 1979).

Several assays for quantification of steroid receptors have been developed through the years. The one most widely used is the dextran-coated charcoal (DCC) method, originally described by Korenman in 1968, but modified several times since then for various hormone receptor determinations (Hähnel & Vivian, 1975; Daehnfeltd & Briand, 1977). Data are usually plotted according to Scatchard (1949), to obtain the quantitative and kinetic characteristics of the binding. This method measures free cytosol receptor sites only, the 8S and 4S binding sites combined. Katzenellenbogen et al (1973) found that an exchange of endogenous hormones was achieved if the temperature at incubation was raised from the normal 0°C to 25-30°C, thus enabling total binding sites to be measured.

Isoelectric focusing in polyacrylamide gel (IFPAG), combined with limited proteolysis, described by Gustafsson et al (1978), is an extensive refinement of the DCC-method, and is used for quantitative assay of free cytosolic 8S receptors.

Other methods have also been used, but they are not discussed here, as they have not been used in these studies.

Biochemical receptor assays in general are time-consuming and include several steps where mechanical, temperature and time factors may considerably affect results (Evans & Hähnel, 1975; Hähnel & Vivian, 1975; Hasson et al, 1981; Wittliff, 1984). Furthermore, the amounts of receptors are related to the concentrations of endogenous hormones (Wittliff, 1984), of proteins (Skovgaard Poulsen, 1981), DNA (Auer et al, 1980), salt (Peck & Clark, 1977), of charcoal (Skovgaard Poulsen, 1981) and to the pH (Sällström, personal communication), all factors capable of considerable variation (Hähnel et al, 1979). Moreover, the glycerol or dithiothreitol added in some laboratories to stabilize the occupied receptor protein may cause variations in results (Philbert & Raynaud, 1973; Hähnel & Vivian, 1975; Wagner & Jungblut, 1980).

Whereas inter-assay variations within the same laboratory is usually within an acceptable range, variations from one laboratory to another tend to be greater, even when the same method is used (Braunsberg & Hammond, 1979; King et al, 1979; Oxley, 1984).

HISTOCHEMICAL LOCALIZATION OF STEROID BINDING SITES

Biochemical methods do not allow morphological localization of the steroid binding proteins. As tissue structures are composed of heterogenous cell populations, which vary in their hormone binding capacities, (Barrows et al, 1980; Silfverswärd et al, 1980a; Castagnetta et al, 1983), many attempts have been made to find histochemical methods for intracellular localization of the steroid binding sites, a type of method for which even small tissue samples would suffice. Several methods have been described based on one of the following two principles:

a. Visualization of the binding sites by means of a specific ligand labeled in such a way, that it can be visible in light microscopy, fluorescence microscopy,

electron microscopy or with autoradiography. The labels can be covalently coupled directly to the ligand, e.g. fluorochromes (Dandliker et al, 1978; Lee, 1978; Barrows et al, 1980; Pertschuk et al, 1980a), or peroxidase (Ghosh et al, 1978; Kopp et al, 1979; Walker et al, 1980; Taylor et al, 1981) or radioactive isotopes (Tchernitchin et al, 1973; Stumpf & Sar, 1982; Gould et al, 1983) or indirectly via antibodies against the ligand (Nenci et al, 1976; Pertschuk, 1976; Mercer et al, 1981). This principle localizes only unoccupied binding sites.

b. Visualization of the binding sites by means of specific labeled monoclonal antibodies against the steroid receptor protein (Greene et al, 1980; Raam et al, 1982; Press & Greene, 1984). Monoclonal antibodies show affinity for either nuclear or cytoplasmic receptors, both occupied and unoccupied.

All histochemical methods visualize the binding sites in the cells but, except the specific antibody technique, provide no information about the character of the binding macromolecule. Furthermore, the steroid binding sites can not be quantified as the results of the measurements may vary according to the thickness of the section, and the fluorescence also to the time lapse after incubation, and to the duration for exposition of the exciting light from the microscope; factors that are difficult to standardize. In addition, the binding of the conjugate may be affected by the temperature and drying.

ESTROGEN AND PROGESTERONE BINDING IN ENDOMETRIOTIC TISSUE AND UTERINE ENDOMETRIUM

ER and PR in normal human uterine endometrium have been extensively studied (Tsibris et al, 1978; Jänne et al, 1979; Martin et al, 1979; Spona et al, 1979; Levy et al, 1980; Neumannova et al, 1983). The ERc level increases during the proliferative phase, reaching a maximum at ovulation, after which it steadily falls throughout the luteal phase to a level below that seen in the follicular

phase. The PRc level too reaches a maximum at midcycle. After ovulation, when the serum progesterone increases, the PRc level decreases (Fig. 2). The amount of steroid receptor varies in different parts of the endometrium, being highest in the functional layer in the fundal region and declining towards the cervix region (Robertson et al, 1971; Tsibris et al, 1981; Cao et al, 1983), and also declining in the basal layer (Watanabe, 1980).

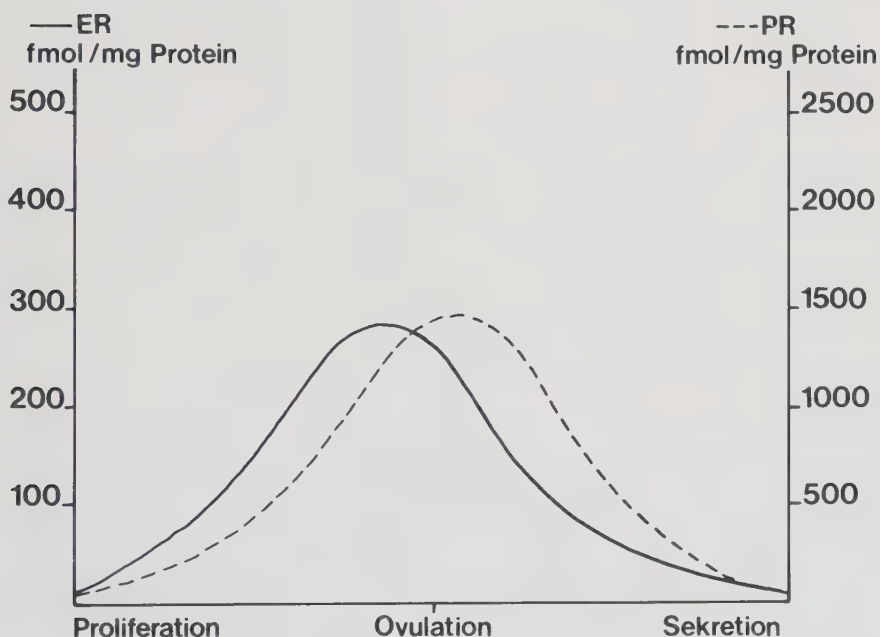


FIG 2

Whether ER and PR occur in endometriotic tissue was considered worth studying, as the endometriotic structures are held to be sensitive to estrogen and progesterone, and as these hormones have been used for therapeutic purposes. Only a few studies on steroid binding in endometriotic samples have been published (Tamaya et al, 1979; Bergqvist et al, 1980; Jänne et al, 1980; 1981; Gould et al, 1983). The results have been somewhat contradictory, but in all studies the ERc and PRc levels have been found to be lower

in endometriotic tissue than in uterine endometrium. In an autoradiographic study, Gould et al (1983) found nuclear estrogen binding sites both in endometriotic tissue and in uterine endometrium. No receptor assays on fine needle aspirates of endometriotic tissue have been reported.

Press & Greene (1984) have presented an immunocytochemical method for demonstration of ER in human endometrium using monoclonal antibodies. No studies have been published on histochemical localization of estrogen and progesterone binding sites in human endometrium or endometriotic tissue using fluorochrome labeled steroid-protein conjugates.

EXPERIMENTAL STUDIES ON ECTOPIC ENDOMETRIUM

One experimental study on humans has been reported (Ridley & Edwards, 1958). This study showed that uterine endometrium (even fragments occurring in the menstrual blood) may be autotransplanted to the peritoneal cavity, grow and respond to exogenous hormones.

A large number of animal experiments have been performed to study the etiology and natural course of endometriosis. In menstrual diversion experiments on monkeys, Scott et al (1953) showed that Sampson's reflux theory is decidedly possible. Most experimental studies have been performed on rhesus monkeys, *Macaca mulatta* (Markee, 1948; Te Linde & Scott, 1950; Dizerega et al, 1980). However, rhesus monkeys are both difficult to obtain and expensive for use in experimental studies. A few studies on induced endometriosis have been performed in rabbits (Jacobson, 1922; Katz & Szenes, 1926; Krichesky, 1943; Merrill, 1966; Schenken & Asch, 1980) and rats (Jones, 1984).

Heterologous transplantation of human tissue has previously only been performed in animals with specific immunodeficient organs, or to animals that have obtained immunosuppressive treatment. The mouse mutant "nude" was de-

scribed in detail for the first time 1966 (Flanagan). The nudes have a congenital thymus aplasia (Pantelouris, 1968) and a pronounced lymphopenia (de Sousa et al, 1969; Wortis, 1974). Because the immune system of the nudes has no self-recognition, it does not reject heterografts (Rygaard & Povlsen, 1969), which renders them suitable hosts for heterologous transplantation.

During the past decade, most types of human tumors have been transplanted into nude mice (Povlsen et al, 1980), and the nudes have been widely used for metabolic (Carrascosa et al, 1984; Emery et al, 1984), immunological (Gorczyński et al, 1984; Pearlstein et al, 1984), radiological (Ikeda et al, 1984; Teicher & Rose, 1984) and pharmacological studies (Povlsen & Rygaard, 1974; Shimotsato et al, 1976; Giovanella et al, 1984; Melvin et al, 1984; Reid et al, 1984; Zaino et al, 1984).

In the only published study of human endometrium and endometriotic tissue transplanted into nude mice (Zamah et al, 1984), endometriotic samples from two women were implanted into two groups of mice (i.p. in one, s.c. in the other), and samples of uterine endometrium from two other women were similarly implanted into two other groups of mice. Some of the mice were treated with estrogen. No parallel transplantations of both tissue types to different sites in the same mouse have been published.

THE PRESENT INVESTIGATION

AIMS

- To compare the histology of endometriotic tissue and uterine endometrium from different sites, exposed to endogenous hormones and obtained simultaneously from the same woman at various phases of the menstrual cycle (I).
- To compare the occurrence and amount of biochemically assayed ERc and PRc in endometriotic tissue and uterine endometrium obtained simultaneously from the same woman at various phases of the menstrual cycle (II).
- To evaluate the specificity of a histochemical method for demonstration of estrogen and progesterone binding in endometriotic tissue and uterine endometrium (III, V).
- To study the binding patterns of the previously evaluated fluorochrome-labeled estrogen and progesterone to endometrium at different phases of the menstrual cycle (IV).
- To compare histochemically the occurrence and localization of specific estrogen and progesterone binding sites in endometriotic tissue and uterine endometrium obtained simultaneously from the same woman (V).
- To investigate whether human endometrium and endometriotic tissue transplanted into nude mice can survive and grow expansively (VI, VII).
- To investigate whether the histological differences between endometriotic tissue and uterine endometrium might be the result of differences in their surrounding milieu (VII).
- To compare the histological effects of various exogenous hormones upon endometriotic tissue and uterine endometrium obtained simultaneously from the same woman and transplanted parallel s.c. into nude mice (VI, VII).

MATERIAL AND METHODS

(Detailed information is provided in the individual papers, I-VII.)

HISTOLOGICAL COMPARISON OF ENDOMETRIOTIC TISSUE AND UTERINE ENDOMETRIUM (I)

Endometriotic tissue was compared histologically to uterine endometrium obtained simultaneously from 28 women. In some cases, endometriotic samples were obtained from more than one site. In all, 47 endometriotic samples were studied. The menstrual cycle day (± 3 days) was determined in each tissue type according to the predominant phase pattern.

Comments: The morphology of endometriotic lesions is often deranged by diffuse bleeding in the stroma and accumulation of hemosiderin-loaded macrophages, ingrowing fibrosis, compression of cystic content, and eventually by progressive atrophy and degeneration of both glands and stroma which frequently results in the absence of intact epithelium. For the dating of endometriotic samples, those parts of the epithelium that were most vital in appearance were used.

The histological pattern of endometrium varies somewhat from site to site within the uterine cavity - differing, for instance, between the functional and basal zones, and between the fundal and isthmus regions and the endocervical mucosa. Glands in the basal zone are usually non-reactive to progestagen influences (Novak & de Lima, 1948). Nor, moreover, are all glands in a given area always in phase (Schweppe et al, 1984). In this retrospective study, we did not accept for inclusion uterine endometrial samples that had been obtained only from the basal zone; and, wherever possible, we included samples that had been obtained from the fundal region.

Novak & de Lima (1948) and Jeffcoate & Potter (1934) found a high frequency of endometrial hyperplasia in their patients with endometriosis. To study the cyclic histological changes in the tissue, we excluded all cases where the endometrium showed pronounced hyperplasia, and included only a few cases where cystic glandular hyperplasia had developed within limited areas, but in which the bulk of the evaluable samples were normal with a menstrual cycle phase pattern which permitted dating.

Since the degree of differentiation may vary in different areas of a section, the dating was based on the most advanced consistent phase pattern according to Wynn (1977). Using the extensive criteria given by Noyes et al (1950) and Wynn (1977), it is possible to define the menstrual cycle day, plus or minus one day. However, because of the limited availability of evaluable endometriotic structures, and as we could not use all recommended variables in the stroma, we set the limits of cycle day definition to plus or minus 3 days.

BIOCHEMICAL ASSAYS OF ERc AND PRc IN ENDOMETRIOTIC TISSUE AND UTERINE ENDOMETRIUM (II)

Endometriotic tissue was obtained at laparotomy from 20 women, uterine endometrium being obtained simultaneously by curettage from 12 of the women. The control material consisted of uterine endometrium from 30 other women.

The IFPAG method was used for the ERc assays, and the DCC method for the PRc assays. The receptor levels were given in fmol bound ligand per mg protein.

Comments: The DCC-method requires relatively large amounts of tissue, (about 0.2-0.4 g wet weight), and the IFPAG method somewhat smaller amounts. As most endometriotic lesions are very small, it is difficult to obtain samples large enough for quantitative receptor assays. The receptor levels are mean values of all cytosol in the tissue

sample, and as endometriotic samples invariably contain some blood, and minor lesions contain both surrounding and ingrowing connective tissue, reliable results will not always be obtained with regard to the receptor quantities in pure endometriotic structures. All the cellular components just mentioned also affect the protein content.

Another assay method requiring smaller sample volumes is needed. In some studies ER has been assayed in fine needle aspirates using DCC (Skovgaard Poulsen et al, 1979), or IFPAG techniques (Gustafsson et al, 1978; Silfverswärd et al, 1980b). We have sampled material from endometriotic lesions in three women using a fine needle. However, since the sparse material consisted mostly of blood, steroid receptor assays on those samples were regarded as irrelevant. Nonetheless, the samples contained sufficient amounts of endometriotic cells to obtain growth of endometriotic tissue when injected s.c. into nude mice (unpublished data).

Other techniques, for which only small samples are needed have been published. Steroid receptor determination by isoelectric focusing has also been performed on fresh tissue sectioned in a cryostat (histoisolectric focusing, Underwood et al, 1983), and Garola & McGuire (1978) have described an hydroxylapatite micromethod for which only 0.05 ml cytosol is needed. In a few studies flow cytometry has been used for ER assay (Raber et al, 1982; Van & Raber, 1984), a technique allowing assay of ER in separate cells. However, none of these methods was available at our laboratories when the study was done.

The synthetic gestagen used for the PR assay was R5020, which is bound with a higher affinity to PR than progesterone (Philbert & Raynaud, 1974; Raynaud, 1977). The binding specificity is higher than that of progesterone, as R5020 is bound only to the 8S component, compared with

progesterone that also is bound to the 4S component. R5020 binding to CR and AR is of low affinity and it is not bound to ER (Raynaud, 1977). Its binding to CBG is minimal (Raynaud, 1977), as it is to other transport proteins. As high concentrations of progesterone may compete with R5020 at PR, we used a constant amount of H³-labeled R5020 and progressively increasing amounts of unlabeled progesterone in five steps.

EVALUATION OF A HISTOCHEMICAL METHOD FOR LOCALIZATION OF SPECIFIC STEROID BINDING (III)

Specific steroid binding in uterine endometrium was histochemically localized, using steroid-albumin complexes labeled with a fluorochrome (estradiol-17 β -bovine serum albumin-fluorescein isothiocyanate, E₂-BSA-FITC, and progesterone-bovine serum albumin-tetramethylrhodamine isothiocyanate, progesterone-BSA-TMRITC) according to Lee (1978). Striated muscle biopsies were used as negative controls. Steroids and non-steroidal hormonally active compounds with known affinity to the receptors were chosen as competitors. Moreover, some procedural steps were evaluated. The homogeneity of the steroid-fluorochrome derivatives was examined, using gel filtration in Sephadex G-25 medium and by dialysis.

Comments: Since the binding specificity of the conjugates was previously insufficiently evaluated, blocking studies were performed on endometrial tissue. Being more homogeneous than most of the more widely used breast cancer tissue, endometrial tissue is well suited for use in large series of consecutive sections for blocking experiments. The relative binding affinities of the different proteins to the various steroids and non-steroidal compounds used are illustrated in Table I.

The histochemical method used does not permit precise measurement of the steroid binding sites, only the rela-

TABLE I In vitro relative binding affinity for specific steroid receptors and plasma proteins, reported in the literature*.

Substance	ER	PR	CR	SHBG	CBG
DES	very high	none	none	none	none
E ₂	high	very low	none	high	none
Tamoxifen	medium high	very low	none	none	none
αDHT	low	medium high	low	very high	very low
R1881	none	very high	medium high	low	very low
Progesterone	very low	high	medium high	very low	high
ORG2058	none	very high	low	none	none
R5020	none	very high	low	none	low
Cortisol	none	very low	high	very low	very high
Dexamethasone	none	very low	very high	none	none
Hydrocortisone	none	very low	low	none	medium high

* (Bonne & Raynaud, 1976; Jänne et al, 1976; Rochefort & Garcia, 1976; Cowan et al, 1977; Lippman et al, 1977a; Lippman et al, 1977b; McGuire & Horwitz, 1978; Raynaud, 1977; Dunn et al, 1981; Pugeat et al, 1981)

tive proportion of fluorescent cells can be calculated. The chosen limit of 10% of the cell population determining whether a sample is positive, has also been used by others (Pertschuk et al, 1979; Rao et al, 1980a). Comparative studies have shown that, if 10-20% or less of the cell population are negative according to a histochemical method, they are usually negative by the biochemical method (Mercer et al, 1980; Nenci, 1981).

Some authors have questioned whether the histochemical methods (except the use of anti-receptor antibodies) and biochemical assay methods indicate the same steroid binding protein (Chamness et al, 1980; McCarthy et al, 1981; Mercer et al, 1981; Penney & Hawkins, 1982; Underwood et al, 1982). The specificity of steroid-fluorochrome conjugates for histochemical techniques has been questioned. However, only a few competition studies have been performed on some conjugates, and results have been contradictory (Dandliker et al, 1978; Daxenbichler et al, 1980; Pertschuk et al, 1980b; Curtin et al, 1982; Fisher et al, 1982).

In some methods the steroids are bound to the fluorescent molecule via a BSA bridge. The presence of fluorescein or BSA covalently linked to the steroid does not interfere with receptor binding (Rao et al, 1980b).

Some workers have also claimed that the conditions used for histochemical methods are in conflict with the kinetic variables used in characterizing free receptor proteins in dilute solution. It has been pointed out that, since the receptor protein is a component of the soluble cytoplasm, it may leach out into aqueous processing media from unfixed cells disrupted by the cutting of frozen sections (McCarthy et al, 1980; Penney & Hawkins, 1982; Underwood et al, 1982). However, the fact is overlooked that the concentration and conformation of the receptor sites in

the target cells are evidently different from those in the supernatant of the tissue homogenate, probably governed by a set of modified kinetic actions, such as those occurring in an immobilized enzyme system. Most investigators who have used this technique have reported fairly reproducible observations with regard to the distribution of estrogen and progesterone binding in the different cell types in the target tissues (Lee, 1979; Mercer et al, 1980). However, the binding of the labeled ligand is strictly localized to structures known to be sensitive to the hormones. Connective tissue, striated muscles, spleen and other tissue types known to be non-responsive to estrogen and progesterone have been shown to be negative references. As the steroid-fluorochrome conjugates bind only to specific structures and the binding pattern changes cyclically during the menstrual cycle, a considerable part of the binding protein must be retained and the binding seems to be specific.

HISTOCHEMICAL LOCALIZATION OF STEROID BINDING IN ENDOMETRIUM (IV)

Histological differences in binding of E_2 -BSA-FITC and progesterone-BSA-TMRITC in consecutive sections of the endometrium during different menstrual phases were studied in samples from 36 women. Histological dating was performed on one consecutive section. Quantitative biochemical assays of ERc and PRc were performed in another part of the tissue sample.

Comments: Since, for ethical reasons, no samples were taken from healthy women, many of the specimens were obtained from women with bleeding irregularities, and for clinical reasons our samples had to be obtained in connection with surgery. However, only samples with normal histological pattern were included. To characterize the menstrual phase of the endometrium, the histological picture was correlated to serum concentrations of estro-

diol-17 β (E₂), progesterone (P), follicle stimulating hormone (FSH) and luteinizing hormone (LH). Our basis for the definition of menstrual endometrium was strictly histological. From this point of view the menstrual changes occur one day in advance of vaginal bleeding.

In this study we accepted a storage time of up to 5 months at -70°C. In our research during the last years, the histochemical method has been repeated on tissue samples after up to one and half a year without any noticeable difference in the binding pattern (unpublished data). Pertschuk et al (1979) found both ER, AR, and well preserved structure in prostatic specimens stored in liquid nitrogen for up to 24 months. Even if some workers have reported a pronounced loss of receptors after a few months of storage, recent publications on steroid receptor assays have shown, that, if the tissue sample is handled correctly, reduction in the amount of receptor is small after one year of storage at -70°C (Hilf et al, 1980; McCarty et al, 1984; Pousette, personal communication).

In several studies the extent of the fluorescence has been used for quantitative measurements of binding proteins. The results of different types of histochemical techniques have been compared with the results of biochemical assays (Lee, 1979; Mercer et al, 1980; Pertschuk et al, 1980b; Taylor et al, 1981; Hanna et al, 1982; Jacobs et al, 1983; O'Connell & Said, 1983; Parl et al, 1984). Although many workers have reported a good agreement between the two techniques, all attempts at quantitative correlations between the results of histochemical and biochemical methods for studies of specific steroid binding proteins are inadequate for two main reasons: first, the assays have to be performed in different parts of a tissue sample which in most cases is heterogeneous; second, the biochemical assay provides information about the mean value of a receptor protein in the cytoplasm of all cells

included in the sample, while the histochemical method indicates the binding to specific cells in a few sections of the tissue sample. However, in view of the occurrence of a qualitative cyclic co-variation of steroid binding, results obtained by the two different assay methods may be of physiological significance. In some studies the histochemical technique has been reported to have a higher predictive value than biochemical assays for clinical response to hormonal treatment (Pertschuk et al, 1979; 1980a). Thus, if the patterns detectable histochemically are used as markers for correctly predicting patient response to endocrine therapy, the technique should prove useful.

HISTOCHEMICAL LOCALIZATION OF STEROID BINDING IN ENDOMETRIOTIC TISSUE AND UTERINE ENDOMETRIUM (V)

Histochemical localization of binding of E_2 -BSA-FITC and progesterone-BSA-TMRITC was performed in 30 endometriotic samples from 21 women. The binding was compared to that in uterine endometrium obtained simultaneously from 14 of the women. The specificity of the binding in endometriotic tissue was studied, using the same series of blocking experiments as that previously performed in uterine endometrium (see paper III).

Comments: The extent of fluorescence could be evaluated, and the binding of the two conjugates in consecutive sections could be compared, but no statistical calculations on the binding extent could be made. Furthermore, a comparison of the extent of the fluorescence in endometriotic samples and uterine endometrium from the same woman was possible. Since cryostate sections were often fragmented and the glandular circumference destroyed, the whole glands could not always be evaluated; consequently, the grading of steroid binding was based on the relative proportion of fluorescent epithelial cells. As the endometriotic samples often contained only a few glands or

destroyed epithelial structures, we chose to grade the binding extent according to whether more or less than half of the epithelial structures were fluorescent.

HISTOLOGICAL EFFECTS IN UTERINE ENDOMETRIUM TRANSPLANTED INTO NUDE MICE AND EXPOSED TO EXOGENOUS HORMONES (VI)

Normal endometrium from 8 women was transplanted s.c. into nude mice, 4 mice per woman. One mouse was treated with PEP, one with MPA, one with danazol and one mouse remained untreated as a control.

Comments: As only grafts of limited size can be successfully transplanted and remain viable, no large specimens were available for interpretation.

The dose of danazol given to animals in different experimental studies has varied widely (Dmowski, 1979). The substance is fairly non-toxic (LD_{50} by the subcutaneous route being >8000 mg/kg), and we chose a high dose to ensure a histological effect. The dosage of PEP and MPA was based on previous experience at the laboratory (unpublished data).

HISTOLOGICAL EFFECTS IN ENDOMETRIOTIC TISSUE AND UTERINE ENDOMETRIUM TRANSPLANTED INTO NUDE MICE AND EXPOSED TO EXOGENOUS HORMONES (VII)

Endometriotic tissue and uterine endometrium, simultaneously obtained from each of six women, were transplanted into 24 nude mice. Samples of both types of tissue from any one woman were transplanted into each of four mice, endometrium in one flank and endometriotic tissue in the other, the mice being given an initial injection of PEP. Two weeks later differentiated treatment was initiated, one mouse receiving PEP, one MPA, one danazol, and one mouse remaining untreated. After 8 weeks of differentiated treatment, all mice were killed, and the grafts extirpated for histological examination.

Comments: As several other studies have shown an unchanged histological pattern in normal tissue transplanted into nude mice (Reed & Manning, 1973; Rygaard, 1974; Holmstrup et al, 1981; Furgyik & Rausing, 1984), we chose the nudes as suitable hosts for transplantation of endometrium and endometriotic tissue. Whereas Zamah et al (1984) grafted endometrium from two women into one group of mice, and endometriotic tissue from two other women into another, we transplanted endometrium and endometriotic tissue from one woman into the same mouse to ensure comparable environmental circumstances for the two types of grafts.

As in the previous study, a somewhat higher taking rate was obtained in grafts exposed to PEP, all mice were given one injection initially before the differentiated treatment was introduced. A similar schedule was used by Potts et al (1974) who primed the animals with E_2 during 6 days before the treatment with danazol was induced in a study of endocrinologic activity of danazol in the rat and the rabbit.

RESULTS AND DISCUSSION

HISTOLOGICAL COMPARISON (I)

In 82% of the cases, a menstrual phase pattern had developed in the endometriotic samples, although the epithelium was often polymorphic in appearance. The histological comparison between uterine endometrium and endometriotic tissue from different sites obtained simultaneously from the same woman revealed a synchronous phase pattern in 54% of all the women; 47% of the endometriotic samples were in phase with the endometrium. Where the lesions were surrounded with thick fibrosis, which had grown into the lesion and completely replaced the stroma, no phase pattern had developed.

Discussion: Our results are in accordance with some others (Roddick et al, 1960; Nieminen, 1962), who found a synchronous phase pattern in endometrium and endometriotic tissue in 60-70% of the cases. The capacity of endometriotic tissue to respond to hormonal influence seems to be dependent on the extent of surrounding fibrosis. This is in agreement with the observations by Cullen (1896), who reported that the less specific stromatic tissue surrounding the glands, the fewer cyclic changes occurred in the glands. He observed that the epithelium was cylindrical when surrounded by specific stroma, but cuboidal or flat when it rested directly against fibrous tissue with no intervening stroma. Our interpretation might explain the results by Novak & Hoge (1958), who found that superficial endometriotic structures in the cervical mucosa often show a true secretory response, while this type of reaction is seldom to be found in endometriotic tissue situated deep in the endocervical myometrium. We found no phase differences in endometriotic lesions from different anatomical sites.

COMPARISON OF RECEPTOR CONTENT (II)

Comparing receptor content in the two types of tissue, the concentrations, both of ERc and of PRc were found to be much lower in the endometriotic tissue than in uterine endometrium. Moreover, ERc were undetectable in 12/20 (60%) of the endometriotic samples, and PRc in 7/9 (78%).

Discussion: These results may be compared to the findings by Kauppila et al (1984), who reported that PRc were undetectable in 6% of the samples and that ERc were undetectable in 70%. When detectable, both ERc and PRc were significantly lower in endometriotic samples than in uterine endometrium from the same patient. In a comparative study on 5 women, who had not received hormonal treatment, Tamaya et al (1979) found that PRc, and to lesser extent ERc, was lower in endometriotic tissue than in uterine endometrium obtained simultaneously.

Dilution of the receptor containing cytoplasm with blood and inappropriate tissue, will make the receptors undetectable. Since we were very keen to obtain pure endometriotic tissue, most tissue samples were scraped from the inside of endometriomas. Histological examination of the samples showed no fibrosis, or only very scant fibrosis, but sometimes rather extensive bleeding. The bleeding results in a dilution effect and also affects the protein concentration in the sample. Moreover, since the sample volume was often small, and had to be diluted for the assay, receptor concentrations being already low might give rise to false low results.

SPECIFICITY OF THE HISTOCHEMICAL METHOD FOR LOCALIZATION OF STEROID BINDING (PAPER III and V)

The results obtained in blocking studies in endometriotic tissue largely agreed with those obtained in uterine endometrium, and indicate a specific binding of medium to low capacity (Table II). There was a pronounced competition between the fluorochrome labeled steroids and steroids with known high specific binding affinity to the steroid receptor.

Discussion: The specificity studies on the binding of E₂-BSA-FITC and progesterone-BSA-TMRITC are difficult to evaluate, as the technique does not permit quantitative but only rough semi-quantitative measurements. To varying degrees, most steroids bind to more than one receptor, and also to other proteins. The relative binding affinity of progesterone is not very high. The weak inhibition of binding of the fluorochrome labeled progesterone to endometriotic tissue exerted by dexamethasone was not very surprising, as it is well known from competition studies on PR, that dexamethasone will bind to PR to some extent (Raynaud, 1977). Since cortisol binds to PR to some extent, a pronounced inhibition of progesterone binding in endometrium was not unexpected (Raynaud, 1977). Why a

TABLE II

	Blocking substances	Blocking effect	
		Endometrium	Endometriotic tissue
E ₂ -BSA-FITC	E ₂	pronounced but incomplete	complete
	DES	complete	complete
	Tam	complete	pronounced but incomplete
	R1881	no inhibition	no inhibition
	5 α DHT	pronounced but incomplete	weak
Progesterone-BSA-TMRITC	ORG2058	pronounced but incomplete	pronounced but incomplete
	R5020	complete	complete
	Dexametasone	no inhibition	weak
	Cortisol	pronounced but incomplete	no inhibition
	5 α DHT	pronounced but incomplete	pronounced but incomplete

similar inhibition of the progesterone binding by cortisol was not seen in the endometriotic tissue, is however enigmatic. The high affinity of the binding sites studied here was well illustrated by the retention of the ligand against prolonged washing. The reproducibility was good, the same binding pattern and binding extent being seen in several consecutive sections.

Critics of histochemistry assert that the number of type I binding sites on the average target cell is insufficient for them to be visualized (McCarty et al, 1980). This assertion is based upon biochemical measurements of tumor cytosol and ignores the fact that type I ER has been demonstrated in various cell organelles and membranes, all of which are discarded in cytosol preparation, but are present in intact tissue sections. Concentrations of ER in these structures might well be visualized by fluorescence microscopy. However, results from some blocking studies indicate that the steroid conjugates used for histochemical methods binds to type II binding sites (Danguy et al, 1981; Mercer et al, 1981).

HISTOCHEMICAL LOCALIZATION OF SPECIFIC STEROID BINDING (IV and V)

When the histochemical technique for localization of steroid binding was used in endometrium, the binding pattern was found to change during the menstrual cycle, being more pronounced in the epithelium than in the stroma in the proliferative phase, and of the same intensity in the stroma as in the glands in the secretory phase, though sometimes even more pronounced in the stroma. The localization of the fluorophores varied with cycle phase, being either basal or evenly distributed throughout the epithelial cells in the proliferative phase, and only apical, or sometimes only basal, in the secretory phase. The binding pattern was similar for both the estrogen and progesterone reagents.

When the histochemical technique was applied to endometriotic structures, the glands were found to be fluorescent to about the same degree as the endometrium when fluorochrome labeled estrogen was used, but to a somewhat greater extent when the fluorochrome labeled progesterone was used. However, no differences in binding pattern according to cycle phase could be found.

Discussion: In most studies on histochemical localization of steroid binding, this has been most pronounced in epithelial structures (Lee, 1981). However, cyclical changes in the binding pattern have not been reported previously. It is unlikely that the differences in binding pattern in the various menstrual cycle phases found in our material, were due to technical factors, and the different pattern in the secretory phase can not be explained by the localization of the nucleus only.

Our results in endometriotic tissue are in accordance with the autoradiographic findings of Gould et al (1983), who found cyclical changes in the nuclear estrogen binding in endometrium, but no changes in the localization of nuclear estrogen binding sites in endometriotic tissue. The extent of the fluorescence in the endometriotic samples in our study could not be related to that of the surrounding fibrosis. It is possible that the amount of steroid binding sites are affected by the fibrosis, but as the method does not permit quantitative measurement of the binding, this effect could not be evaluated. However, the results do not indicate a lower degree of progesterone binding in endometriotic tissue than in the endometrium.

TRANSPLANTATION TO NUDE MICE (VI and VII)

The two types of tissue could be transplanted into nude mice with an acceptable taking rate. Both types of tissue changed histologically according to the hormonal treatment given, and control grafts, that were not exposed to any

exogenous hormones, lost their phase pattern. In all treatment groups the stroma in the grafts was gradually replaced by loose fibrous tissue. However, the growth of fibroses differed in the various treatment groups: a thick capsule surrounding the graft with mostly well preserved stroma was often found in the PEP treated mice, a thin capsule and diffuse loose fibrous tissue replacing the specific stroma was often found in the MPA treated mice and a thin capsule and a cellular specific stroma was often found in the danazol treated grafts. After some weeks in the same milieu, no obvious histological difference could be seen between the two tissue types both of which now had the appearance of human endometriotic tissue with an irregular glandular pattern. When no residual stroma surrounded the glands, they became narrow, the epithelium flat and the glands seemed to degenerate.

Discussion: In the first study (paper VI) the growth time for the grafts was limited by the poor condition and short survival of the mice. The initial injection of estradiol, the somewhat larger size of grafts implanted, and the better condition of the mice, may have contributed to the higher taking rate and viability of the grafts used in the second study (paper VII). The initial PEP injection may have contributed to the proliferative pattern seen even in the control grafts and to the decidual reaction in the MPA-treated grafts.

Our interpretation that the stroma is of fundamental importance for the glandular appearance, is supported by the report by Hesselberg et al (1918), who performed auto and homoiotransplantations of uterine tissue into the ear and the abdominal wall in guinea pigs.

The fibrous tissue replacing the specific stroma appeared to have its origin in the mouse, as it seemed to continue from the surroundings and often bulged into the stroma.

Isolated areas of fibrous tissue were not found in the grafts. Our interpretation is supported by the report by Warenius (1980) who, when using labeled anti-mouse and anti-human sera, found that human tumors heterotransplanted into nude mice acquire a mouse stroma. Findings of ingrowing fibrous tissue have been reported in other studies (Merenda et al, 1975; Kim et al, 1978). Povlsen & Rygaard (1971) reported that, although a loss of stroma was noted in the tumors, their general structure was preserved.

Hormonal treatment of experimental endometriosis has been performed previously. Scott and Wharton treated monkeys with diethylstilboestrol (1955), progesterone (1957; 1962), testosterone (1959) or norethindrone (1962); they found that estrogen treatment induced hyperplasia, and that constant estrogen and intermittent progesterone resulted in local growth. Neither testosterone, norethindrone nor progesterone used on its own, caused any atrophy of the induced endometriosis.

Our results are in agreement with those of Zamah et al (1984), who injected endometriotic tissue and uterine endometrium, respectively, into nude mice. Half of the mice were treated with estradiol cypionate. Untreated endometriotic grafts mainly showed stroma and necrotic acini at extirpation, while the estrogen treated grafts had grown, were well vascularized and the lining of the glands was highly variable. The endometrial grafts showed considerable variation in glandular size, and glands in the estrogen supplemented grafts were larger than the unsupplemented ones.

The effect of danazol found here differed from the histological effect described in clinical material (Greenblatt et al, 1971; Floyd, 1980; Jeppsson et al, 1984). Mice do not have SHBG (Wenn et al, 1977), but their AFP possesses

high binding affinity for estrogens (Uriel et al, 1972; 1975). However, their AFP does not bind testosterone (Uriel et al, 1972), and as danazol is a derivative of testosterone, a binding to AFP may hardly explain the aberrant effect of danazol on the grafts. On the other hand Bevan et al (1984) means that it seems likely that a major site of action of danazol in humans is a reduction of SHBG synthesis within 1-2 weeks allowing greatly increased concentrations of free testosterone to be biologically available to the endometrium. Moreover, danazol displaces testosterone from SHBG (Nilsson et al, 1982).

The effect of danazol on ectopic endometrium has been reported in two previous experimental studies. Hahn et al (1983) performed autotransplantation of endometrium to the peritoneum of rabbits. They found that untreated grafts increased in weight, and daily oral administration of danazol did not impair the growth until after 8 weeks of treatment, when the weight of the implants and the number of endometrial glands had reduced and a thinning of the endometrial stroma was seen. Jones (1984) found that an luteinizing hormone releasing hormone (LRH)-agonist rapidly inhibited the growth of explanted endometrium in rats, but neither levonorgestrel nor danazol caused any regression of the growth. Thus our results are in agreement with those of Hahn et al (1983) and of Jones (1984) who found that the inhibitory effect on the growth of ectopic endometrium caused by danazol is uncertain and at least slow.

Squamous metaplasia was observed in untreated endometrial grafts from two women, one graft extirpated after 10 days, the other after 17 days. Foci of squamous metaplasia reminding of the metaplasia in the grafts are sometimes seen in endometrial hyperplasia and endometrial cancer (Dallenbach-Hellweg, 1981). This type of metaplasia has also been reported in prostatic tumors transplanted into

nude mice (Reid et al, 1978). Dallenbach-Hellweg (1981) regarded the appearance of the nodules of squamous epithelium as a reaction to elevated levels of estradiol. However, in our cases, as well as those reported by Reid, the grafts were not exposed to exogenous hormones. As the estradiol level in nude mice is low, an estrogen overstimulation may hardly explain the squamous metaplasia, the cause of which is thus unknown.

GENERAL DISCUSSION

Two mutually opposing growth factors seem to affect the morphology of endometriotic lesions, namely an expansive growth of the endometriotic structures into their surroundings, and a progressive growth of surrounding fibrotic tissue encapsulating and invading the lesions.

An expansive growth of endometriotic lesions have been regarded as hormone dependent. Endometriotic tissue seems to contain specific binding sites for estrogen and progesterone, but with biochemical assays, steroid receptors were undetectable in many cases, probably, at least partly, because the assay techniques were inappropriate for that type of tissue. ERc and PRc could not be detected in 60% and 78%, respectively, of the cases.

Although tissue sampling is carefully performed, blood and fibrous tissue will be included to varying extent and affect the receptor levels assayed. Such problems emphasize the need of a valid histochemical method for steroid receptor localization. When these studies were performed, only one histochemical method demonstrating steroid binding sites was available. Using this method, the results showed, however, that endometriotic tissue and uterine endometrium bind E_2 and progesterone to a comparable extent. Even if the binding sites found may not be identical to the conventional receptor protein, the

blocking studies, and the cyclical variations in the binding pattern found, indicate that the steroid binding is specific. During recent years, more than one type of specific estrogen binding proteins have been identified, for example in pancreas, prostate, breast cancer and meningiomas (Katsumata & Goldman, 1974; Sutherland et al, 1980; Pousette et al, 1982; Ekman et al, 1983; Schwartz et al, 1984). On the basis of these findings, it has been suggested that there might exist multiple types of steroid binding sites, all of which may be needed for optimal tissue hormone response (Mercer et al, 1980).

However, in only 54% of the cases, was the histological cycle phase pattern in endometriotic tissue similar to that in uterine endometrium. Thus, though endometriotic tissue often responds to endogenous hormones in the same way as uterine endometrium, in about half the cases no response is obtained. One explanation to the lack of biologic response to the surrounding hormonal milieu is defect receptors. Today no methods are available that can give information about whether the steroid binding proteins assayed are biologic active.

Another possible explanation is that the surrounding factors influence the growth of the endometriotic structures. In order to study the influence of surrounding factors upon the histological pattern in uterine endometrium and endometriotic tissue, these two tissue types were transplanted into the same milieu subcutaneously in nude mice. Also their response to exogenous hormones during the same conditions was studied. We found no difference in the extent of fibrosis surrounding and invading the two types of tissue. Moreover, the changes in glandular pattern, and the epithelial polymorphism seen when the specific stroma disappeared, were the same in samples obtained from endometriotic lesions and uterine endometrium. Moreover, the histological changes, observed

after administration of exogenous hormones, were similar in the two types of tissue; with some exceptions estrogen induced a proliferative pattern, progesterone an inactivation and sometimes atrophy of the glands while the glands during danazol treatment had a vital appearance without atrophy. Our results indicate that the specific stroma seems to have a decisive effect on the histological pattern of epithelial structures both in endometrium and in endometriotic tissue. Whether the negative effect of ingrowing fibrosis depends on disturbed blood supply, and thus on nutrition, or disturbance of some kind of growth-promoting factors in the specific stroma, is not known.

Thus, when the two types of tissue were transplanted into nude mice, the histological pattern developed in the same direction, and after some weeks in the new milieu, the source of the two types of tissue could no longer be distinguished. The histological pattern in both untreated tissues chiefly resembled that of endometriotic tissue with its polymorphic appearance.

The results of the present study indicate that the histological and functional differences between endometriotic tissue and uterine endometrium depend, at least partly, on differences in the surrounding milieu.

CONCLUSIONS

From the findings here the following conclusions may be drawn:

1. The histological pattern in endometriotic tissue is often polymorphic, though a menstrual cycle phase can often be distinguished, corresponding to that in the uterine endometrium in about half of cases. The phase pattern is often similar in various endometriotic lesions in the same case.

2. The biochemically assayed quantities of ERc and PRC are lower in endometriotic tissue than in uterine endometrium, and are often undetectable.
3. The histochemical method used indicates a specific binding of fluorochrome labeled E_2 and P in endometriotic tissue and uterine endometrium.
4. The specific binding of fluorochrome labeled E_2 and P in uterine endometrium changes cyclically during the menstrual phases, being most pronounced in the epithelial structures during the proliferative phase.
5. There is no obvious difference in the extent of specific binding of fluorochrome labeled E_2 and P in endometriotic tissue and uterine endometrium, but no cyclic variations during the menstrual phases can be identified in endometriotic samples.
6. Human endometriotic tissue and uterine endometrium can survive, but does not grow expansively after transplantation into nude mice.
7. Endometriotic tissue and uterine endometrium adapt the same histological pattern when transplanted into the same milieu in nude mice.
8. The histological effect of exogenous hormones is the same in uterine endometrium and endometriotic tissue transplanted into nude mice.

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Human Endometrium and Endometriotic Tissue Obtained Simultaneously: A Comparative Histological Study

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Summary: A comparative study on the histological appearance of endometrium and endometriotic tissue obtained simultaneously has been performed. In all, 47 endometriotic samples from 28 patients were examined. In 15 cases, samples from more than one endometriotic lesion were included. The endometriotic tissue had developed a distinctive menstrual phase pattern in 23 patients (82%). In 16 of these patients (70%), the phase pattern was found to be synchronous (± 3 days) in the two tissue types. In seven cases (30%), it varied inconsistently as compared with the endometrial phase. In the remaining five cases (18%), no menstrual phase pattern was found in the endometriotic specimens in spite of a well-defined phase in the endometrium. When biopsy specimens from different endometriotic lesions in the same patient showed a menstrual phase pattern, the phase did not differ significantly among the specimens. Although a menstrual phase pattern could be recognized in the endometriotic tissue samples in most cases, a considerable variability in glandular and epithelial cell morphology was frequently found. **Key Words:** Endometriosis—Endometrium—Histology—Menstrual phase.

The histological resemblance between endometriotic tissue and uterine endometrium is well known (1-7, et al.). It is generally assumed that the responsiveness of endometriotic lesions to sex hormones is qualitatively the same as that of the endometrium and that the histological variations in the two types of tissue during the menstrual cycle are synchronous. A similar histological menstrual phase pattern in endometriotic tissue and uterine endometrium obtained simultaneously from the same patient has been demonstrated in previous studies (8-10). On the contrary, results indicating a deficient response to progesterone in endometriotic tissue have also been reported (9,11). This is in accordance with

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studies of the steroid receptor content demonstrating a lower level of both estrogen and progesterone receptors in endometriotic tissue than in the corresponding endometrium (12,13).

The aim of the present study was to compare the histological appearance of endometriotic tissues from different locations and to compare these with that of uterine endometrium obtained simultaneously from the same woman, with special regard to the histological menstrual phase pattern.

MATERIAL AND METHODS

Selection of the material

Routine sections from endometriotic tissue and endometrium, obtained simultaneously from 67 women operated upon consecutively, were reviewed histologically. All women were premenopausal. None had been on hormonal therapy or used any other medication within half a year prior to the operation.

The specimens had been fixed in 10% formalin and embedded in paraffin. Sections had been stained with hematoxylin-eosin. All routine sections from the endometrial and endometriotic tissues were scrutinized. A prerequisite for an endometriotic lesion to be accepted was the presence of at least one well-preserved gland surrounded by specialized stroma. Using this criterion, 28 patients (ages ranging from 23 to 50 years, mean age 35 years) were accepted. The histological specimens from the endometrial tissue invariably contained several glands and were all judged adequate for determining the menstrual phase.

Seven patients had undergone hysterectomy because of suspicion of leiomyomas; some of them had experienced bleeding irregularities and/or dysmenorrhea. Eighteen women (age 23–45 years) were eumenorrheic, seven (age 30–50 years) had a history of irregular bleeding, and in three (age 29–40 years), the bleeding pattern was unknown.

In all, 47 endometriotic samples were included in the study. Thirty-six specimens had been obtained at laparotomy, four at laparoscopy, five had been taken from the vaginal pouch, and two from an abdominal scar endometriosis. The various locations of the endometriotic lesions are given in Table 1. Endometriotic tissue had been derived from one location in 13 cases, from two in 12 cases, from three in two cases, and from four in one case. In the cases in which the dominating lesion was a large endometriotic cyst, glands with specific stroma were invariably present in the cyst wall or its neighborhood. In two samples, only a single endometriotic gland with accompanying specific stroma was available for interpretation; in the remaining 45 samples, several glands were present.

Endometrial tissue from the functionalis, in most cases from the fundal region, had been obtained simultaneously by endometrial curettage (21 cases) or by hysterectomy (seven cases).

Histological examination

The histological sections were examined according to a standardized protocol containing several parameters listed in Tables 2, 3, and 4. Determination of the menstrual phase was performed according to some parameters suggested by Noyes et al., Dallenbach-Hellweg, and Robertson (14–16). The parameters used are given in Table 2. The dating was based on the dominating phase pattern of the sample. Because of the great variability, the height of the epithelial cells was

TABLE 1. Anatomical localization of 47 endometriotic samples from 28 patients and their histological menstrual phase compared with that of the corresponding endometrium

Patient no.	Endometriosis		Endometrium
	Anatomical localization	Histological phase pattern	Histological phase pattern
1	L ovary ^a	Inconclusive	EP
2	R ovary	EP	EP
	R ovary	Inconclusive	
	Vermiform appendix	EP	
3	Peritoneum	CD 16	MP
4	Peritoneum	MP	MP
5	R ovary ^a	LP	MP
	Peritoneum	LP	
6	Peritoneum	MP	MP
7	R ovary ^a	CD 22	LP
8	R ovary	LP	LP
	Peritoneum	MP	
9	Scar in the anterior abdominal wall	MP	LP
	Scar in the anterior abdominal wall	MP	
10	L ovary ^a	LP	LP
	R ovary	LP	
11	L ovary ^a	Inconclusive	CD 16
12	Peritoneum	CD 16	CD 16
13	R ovary ^a	Inconclusive	CD 16
	L uterine tube	Inconclusive	
	L ovary ^a	CD 20	
	Peritoneum	CD 18	
14	Vagina	CD 15	CD 16
	Vagina	CD 15	
15	R ovary	CD 27	CD 17
	R ovary	CD 27	
16	L ovary ^a	CD 17	CD 17
	L ovary	CD 20	
	R ovary ^a	CD 17	
17	Peritoneum	CD 17	CD 17
	Peritoneum	CD 17	
18	Peritoneum	Inconclusive	CD 17
19	Vagina	CD 16	CD 17
	L ovary ^a	CD 18	
20	Vagina	CD 18	CD 19
	Vagina	Inconclusive	
21	Peritoneum	Inconclusive	CD 20
22	L uterine tube ^a	CD 20	CD 21
23	R ovary ^a	MP	CD 23
	R ovary ^a	MP	
24	L ovary ^a	CD 19	CD 23
25	L ovary	Inconclusive	CD 24
	L ovary	Inconclusive	
26	L ovary	CD 25	CD 25
	L ovary ^a	Inconclusive	
27	R ovary ^a	CD 25	CD 26
28	L ovary	EP	CD 26

CD, cycle day; L, left; R, right; early proliferative (EP) = CD 3-7; mid-proliferative (MP) = CD 8-10; late proliferative (LP) = CD 11-15. In the secretory phase the cycle day is given with ± 3 days.

^a Glands plus cyst >3 cm diameter.

TABLE 2. *Histological parameters for dating the menstrual phase in endometriotic and endometrial samples obtained simultaneously from the same patient*

Height of epithelial cells
Occurrence of pseudostratified nuclei
Occurrence of mitoses
Distribution of glands
Form of glands
Width of glands
Occurrence of vacuoles
Localization of vacuoles
Predecidual reaction in the stroma

measured at 10 different places by use of an ocular micrometer, and the median was calculated. A median cellular height of less than 15 μm was regarded as "low," 15 μm or more as "high." Cells cut obliquely were not measured. The presence of mitotic figures in epithelial cells was registered. Since mitoses do not occur in all glands during the proliferative phase, the absence of mitoses in samples containing less than five glands was considered nonsignificant. Intraluminal secretion, stromal edema, spiral arterioles, stromal mitoses, fibrin deposits in vessels and stroma, and leukocytes are often useful for the dating of the endometrium. Since endometriotic lesions are often small and the stroma deranged by

TABLE 3. *Histological parameters used for the estimation of glandular and epithelial cell polymorphism in 47 endometriotic and 28 endometrial samples obtained simultaneously from 28 patients (n = number of specimens)*

	Endometrium		Endometriosis	
	n	%	n	%
Distribution of glands				
Even	24	86	7	15
Uneven	3	11	15	32
Not judgable	1	4	25	53
Character of epithelium				
Even in height	22	79	12	26
Uneven in height	6	21	35	74
Nuclear polarity				
Regular	1	4	23	49
Irregular	27	96	24	51
Localization of nuclei				
Regular	22	79	21	45
Irregular	6	21	26	55
Nuclear form				
Round/oval	24	85	22	47
Long, narrow	1	4	7	15
Variable	3	11	18	38
Density of chromatin				
Homogenous	28	100	43	91
Variable	0	—	4	9

TABLE 4. Occurrence of bleeding, chronic inflammation, and fibrosis in 47 endometriotic samples

	Endometriotic samples		Endometriotic samples with distinctive phase pattern	
	no	%	no	%
Bleeding				
Sparse	23	49	16	70
Pronounced	13	28	10	77
Absent	11	23	11	100
Chronic inflammation				
Present	42	89	32	76
Absent	5	11	6	100
Fibrosis				
Present	32	68	24	75
Absent	14	30	13	93
Not judgable	1	2	1	100

bleeding, inflammation, and fibrosis, these parameters were judged as unreliable for dating the menstrual phase and consequently disregarded.

Histological parameters used to evaluate the glandular and epithelial polymorphism in the two types of tissue are given in Table 3.

Since bleeding, inflammatory reactions, and fibrosis might be of interest for the determination of phase pattern in the endometriotic samples, an attempt was also made to estimate these parameters (Table 4).

The endometriotic samples were always examined before the endometrial sections. The examination was performed without prior knowledge of the cycle day or serum hormone levels. In cases where specimens from more than one endometriotic lesion were obtained, an approximated cycle phase of the endometriotic tissue in that patient was estimated from the phase pattern in the separate lesions. This determination was based only on specimens showing a distinctive phase pattern, whereas samples from the same patient having inconclusive patterns were disregarded in this respect.

When comparing the cycle phase pattern in the two tissue types, a ± 3 -day difference was considered as synchronous.

Determination of serum hormone levels

In 20 cases, blood samples were drawn the morning of the operation day or the following morning and the serum levels of estradiol-17 β and progesterone were assayed radioimmunologically (17).

RESULTS

The menstrual phase could be determined histologically in endometrial samples from all 28 patients. In 23 cases (82%), the phase could also be determined in endometriotic specimens. In the remaining five patients (Patients no. 1, 11, 18, 21, and 25), no clear phase pattern was found in the endometriotic samples, but in four of these patients, samples were obtained from only one location. The phase determined in samples from different endometriotic lesions derived from

the same patient is presented in Table 1. In 11 samples from different locations in nine patients, the endometriotic tissue showed no clear histological phase pattern, and these samples were classified as inconclusive. In 16 women (70% of the cases where the endometriotic samples showed a phase pattern), the menstrual phase was synchronous in the two types of tissue (Table 1, Fig. 1), whereas in the remaining seven cases the approximated phase in the endometriotic specimen varied inconsistently as compared with that in the endometrium (Fig. 2).

In those cases in which samples from multiple endometriotic lesions were avail-

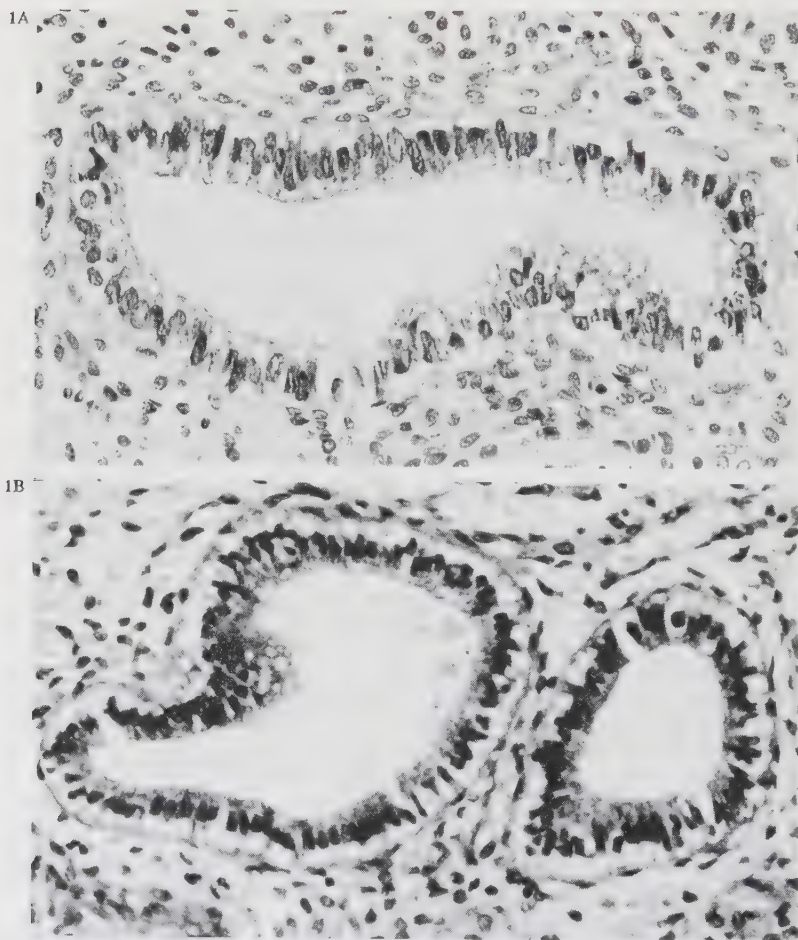


FIG. 1. A: Endometrium (corresponding to the 18th cycle day). B: endometriotic tissue (corresponding to the 17th cycle day) obtained simultaneously from the same patient ($\times 400$).

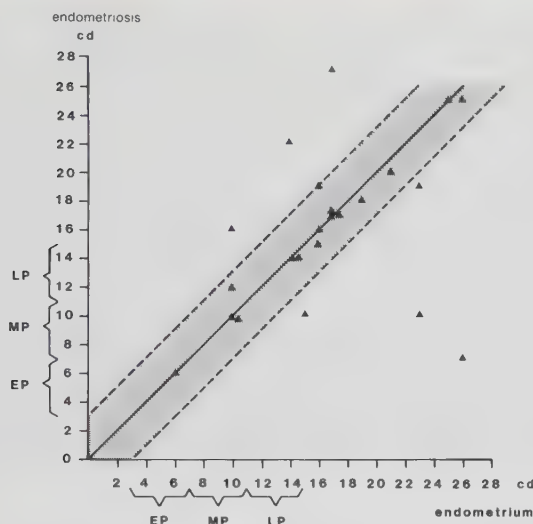


FIG. 2. The correlation between the histological cycle day (cd), in the endometrium and the approximated cd in the endometriotic lesions in 23 patients from whom endometriotic samples, showing a distinctive menstrual phase pattern, were obtained. The shaded area indicates a difference of ± 3 days. EP, early proliferative; MP, midproliferative; LP, late proliferative.

able from the same patient, the cycle day, determined histologically, varied by less than 3 days between different locations.

Histological characteristics indicating structural heterogeneity of the endometrium and the endometriotic tissue are presented in Table 3.

Endometrial polyps were found in six cases. Although a tendency toward cystic glandular hyperplasia had developed in the endometrium in five cases, the phase pattern could still be determined in these cases. Adenomyosis was diagnosed in five of seven women who had undergone hysterectomy. Leiomyomas were verified in five of the seven extirpated uteri. The endometriotic samples from all these patients did not differ from those in the other cases with respect to phase pattern or to other histological parameters studied.

Although dating of the menstrual phase in an endometriotic lesion was possible in most cases, a considerable variability in glandular and epithelial cell morphology was frequently observed (Table 3). The glands were usually more irregularly-outlined and varied in size as compared with the endometrium. Narrow and dilated glands often occurred together (Fig. 3). The epithelium of the glands was less uniform as compared with the endometrium, often showing varying degrees of cytologic polymorphism (Fig. 4). The morphology and chromatin distribution of the nuclei often varied within the same gland. Loss of polarity was also sometimes seen (Fig. 5).

Histologically, the endometriotic lesions were frequently surrounded by a mantle of fibrosis (Table 4) which, to varying degrees, had replaced the specialized

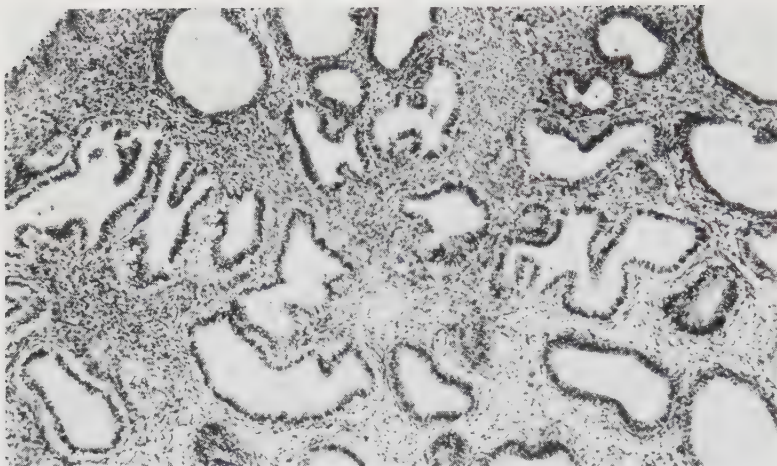


FIG. 3. Endometrial glands of different shape and degree of dilatation ($\times 100$).

stroma. In such instances, the epithelium was most often well preserved, but no phase pattern had developed.

The serum hormone levels of estradiol-17 β and progesterone corresponded in most cases to the histological phase pattern seen in the endometrium. In two cases, however, (Patients no. 16 and 20), the progesterone levels were low, whereas the histological pattern in the endometrium corresponded to the 17th and the 19th cycle day. The menstrual bleeding pattern did not seem to influence the degree of synchronism of the phase pattern in the two types of tissue.

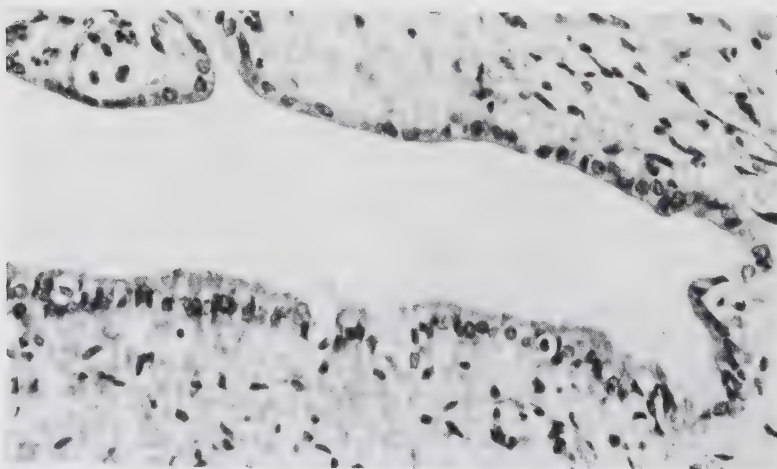


FIG. 4. An endometrial gland lined by epithelial cells with varying height ($\times 400$).

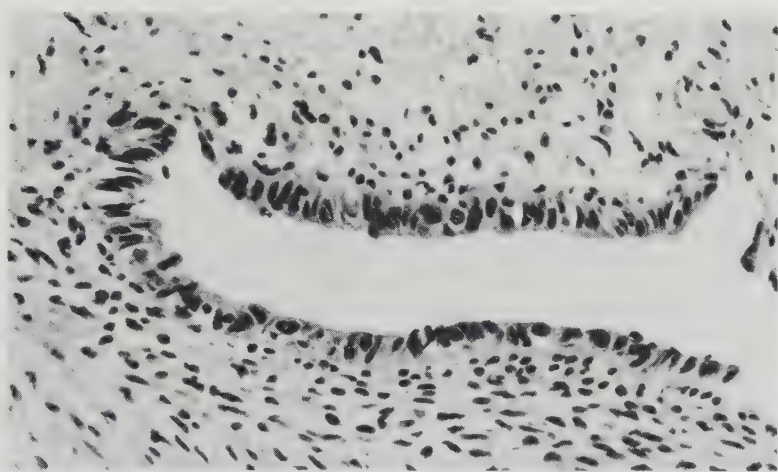


FIG. 5. Cellular and nuclear polymorphism in an endometriotic gland ($\times 400$).

DISCUSSION

This comparative study is based on 28 patients from whom histologically evaluable biopsy specimens from endometrium and endometriotic tissue had been obtained.

Because of the heterogeneous histological pattern found, especially in the endometriotic tissue, we considered a menstrual phase pattern in the two types of tissue with ± 3 -day difference according to Noyes (14) to be synchronous. Assuming a ± 2 -day error in the dating of the secretory phase of the endometrium, Johannisson et al. (18), using the luteinizing hormone peak as a marker for ovulation, found that endometrial biopsy specimens could be correctly classified in 81% of the cases.

There was a good correspondence in cycle phase between the two types of tissue in 16 patients (70% of the cases in which the endometriotic samples showed a phase pattern). In four of the cases with multiple endometriotic lesions, one or two samples in each case showed an inconclusive phase pattern, the remaining having a well-developed phase. In seven cases (30%), the endometriotic tissue had developed a menstrual phase pattern, which, however, was not synchronous with that of the corresponding endometrium. There were no differences between these seven cases and the remaining cases with respect to mean age of the patients, endometrial menstrual phase, degree of fibrosis and bleeding in the endometriotic lesions, or anatomical localization of these lesions. In five of the remaining patients (18% of all cases), no specific phase pattern was found in the endometriotic samples.

Nieminen (10) compared the cycle phase in 96 specimens of endometriotic tissue from different anatomical localizations with the menstrual phase of the patient. However, the endometrial phase was determined histologically only in 28 cases; in the others, the cycle phase was determined on the basis of anamnestic

information or the presence of a fresh corpus luteum at operation. In 64 cases (67%), the endometriotic tissue corresponded to the estimated cycle phase, but it is not clear in how many of these 64 cases the endometrium had been histologically investigated and no details about the comparison are presented.

The irregular and polymorphic appearance of the endometriotic tissue may account for some of the difficulties in determining the cycle phase in some samples. It may be one explanation for the discrepancy in phase pattern sometimes found between the endometriotic and endometrial tissues in the same patient. In this connection it may be worthwhile mentioning the ultrastructural study performed by Schweppe and Wynn (19). They found it possible to date the endometriotic tissue according to criteria similar to those used for dating the uterine endometrium. However, certain features, such as giant mitochondria and nuclear channel systems, that are regarded as characteristic of the epithelium of normal uterine endometrium, could not be detected in the endometriotic tissue.

The irregular morphology of the glands and the polymorphic appearance of cells and nuclei found in the endometriotic specimens could be due to nutritional disturbances such as alterations in blood supply or to abnormalities in the response to hormonal stimuli. Many previous reports have also pointed out a great variability in the histological pattern of endometriotic tissue (1,6,19–21). Novak (20) says that some ectopic endometrial glands that have an inactive appearance have the same characteristics as the basal stratum of the endometrium that shows inconspicuous cyclical changes. In a study comparing adenomyosis, endometriosis, and endometrium, Dallenbach-Hellweg and Grumbrecht (11) found that the response of the endometriotic lesions to endogenous hormones varied according to the anatomic localization of the lesions. Novak (9) also pointed out this variation in a study on cervix endometriosis. We were not able to confirm the influence of the anatomical localization on the histological pattern.

Seitz (22) believed that the inactive pattern found in endometriotic structures surrounded by fibrous tissue was due to the higher pressure inside the fibrotic capsule. In our series, dense fibrous adhesions surrounding the endometriotic lesions were found at operation in 18 cases. No correlation was found, however, between the occurrence of fibrosis surrounding the endometriotic lesion and the occurrence and degree of glandular and epithelial polymorphism in the lesion. In areas where the fibrosis had completely replaced the specific stroma, the epithelium did not show any phase pattern.

It is probable that the degree of fibrosis is related to the age of the lesion (20). The anatomical site (9,11) of the lesion may also play a role in this respect. Furthermore, it is possible that endometriotic lesions of different anatomical localization may differ with respect to histogenesis and degree of morphologic and functional differentiation.

In conclusion, this study has shown histological patterns in endometriotic structures indicating a response to hormonal stimuli. Although the phase pattern in the two types of tissue was found to be synchronous in most cases, the changes in the endometriotic tissue did not always parallel those in the endometrium and had sometimes an atypical appearance.

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ESTROGEN AND PROGESTERONE CYTOSOL RECEPTOR CONCENTRATION IN ENDOMETRIOTIC TISSUE AND INTRAUTERINE ENDOMETRIUM

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Abstract. Cytosol estrogen (ER) and progesterone (PR) receptors were assayed in peritoneal and ovarian endometriotic tissue from 20 patients. In 12 of the cases the intrauterine endometrium was assayed as well. The control material consisted of normal endometrium from 30 women. The receptor content of the endometriotic tissue was less than that of the endometrium of the same patient. In most cases no receptors could be detected (12/20 cases ER-, 7/9 cases PR-) irrespective of the phase of the menstrual cycle. The endometrial receptor concentrations were that same in patients with endometriosis as in the control group. There were no differences in binding characteristics of the receptors in the two tissue types. It is concluded that endometriotic tissue contains lower a concentration of cytoplasmic ER and PR than the normal endometrium.

Ever since its first description by Sampson in 1921 (11) endometriosis has been regarded as the occurrence of ectopic endometrium. The ectopic tissue has been thought to react to hormonal stimuli in the same way as the endometrium. However, certain discrepancies in the histological appearance of the endometriotic tissue and the endometrium have suggested differences in the response of the ectopic tissue to hormonal stimuli (14).

Several studies (6, 13) have shown that the normal uterine endometrium contains detectable concentrations of estrogen (ER) and progesterone (PR) receptors, which vary during the cycle due to the effects of steroid hormones. During the late follicular phase, ER concentration is maximal and prior to and immediately after ovulation PR reach their maximum concentration due to the stimulatory effect of estradiol-17- β . During the luteal phase, cytoplasmic ER and PR concentrations both decrease rapidly due to the action of progesterone on estradiol receptor synthesis and/or inactivation.

However, very little is known about the preconditions for the endometriotic tissue to react to hormonal influences. This study was undertaken to investigate whether there are any differences in cytoplasmic ER and PR concentration in normal intrauterine endometrium and in endometriotic tissue.

MATERIAL AND METHODS

Patients and tissue samples. The study group consisted of 20 patients (age 33 ± 9 years) (mean $\pm$ SD) with endometriosis. Nineteen patients had endometriosis affecting ovaries and/or peritoneum, 14 of whom had endometriomas ≥ 3 cm. One had endometriosis only in a cicatrice in the abdominal wall. At laparotomy, endometriotic tissue was excised from the peritoneum or the ovarian surface or scraped with a knife from the inner surface of the endometriomas. Histologic examination confirmed endometriosis in all cases. Uterine endometrium was obtained by curettage in 12 cases in conjunction with laparotomy.

The control group consisted of 30 women. In 17 subjects (age 42 ± 7 years) endometrium was obtained from the proximal two-thirds of the uterine cavity after hysterectomy (group I). Most patients were operated upon because of leiomyomas. In 13 patients (age 42 ± 8 years) endometrium was obtained by curettage because of menometrorrhagia (group II). Only patients with histologically normal endometrium were accepted.

None of the patients in the two groups was receiving hormonal therapy. All women were menstruating. Based on the histologic appearance and hormone levels, the material was divided into either follicular or luteal phase.

Blood samples were drawn into tubes containing EDTA at 8 p.m. on the day of operation or the day after. The blood was centrifuged and the plasma stored at -20°C until analyzed for estradiol-17- β (E_2), progesterone (P), follicle-stimulating hormone (FSH) and luteinizing hormone (LH). All hormones were determined with conventional radioimmunoassay techniques (15).

Processing of the tissue. Preparation of the tissue samples was performed consistently by one of us immediately after removal. The specimen was freed from inappropriate tissue, rinsed in ice-cold isotonic saline to remove mucus, blood and clots, placed in a dry plastic box on dry ice for up to 2 hours during transportation and then stored at -70°C . The receptor assay was performed within 2 months.

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Table I. Study group: Patients with endometriosis. Plasma levels of estradiol (E_2), progesterone (P), follicle-stimulating hormone (FSH), and luteinizing hormone (LH), cytoplasmatic concentration of estrogen (ER), and progesterone (PR) receptors, and histologic classification of the tissue samples.

Case No.	Age	C. d.	Plasma				Endometrium			Endometriosis		
			E ₂ pmol/l	P nmol/l	FSH μg/l	LH μg/l	Histologic picture	ER fmol/mg	PR prot	Type	ER fmol/mg	PR prot
Follicular phase												
12	21	11	170	< 2	2.6	1.6	—	—	—	ovarian	ND	—
14	30	15	340	2.2	3.0	1.6	—	—	—	endometrioma	ND	—
15	25	8	115	< 2	1.1	1.0	proliferative	ND	—	endometrioma	6	—
21	43	7	120	< 2	3.5	< 0.5	proliferative	19	—	peritoneal	3	—
19	33	5	340	1.0	3.9	3.4	—	—	—	endometrioma	ND	—
25	29	7	270	< 2	2.8	2.3	proliferative	50	780	peritoneal	2	61
2	33	—	330	4.1	1.1	2.6	—	—	—	endometrioma	ND	—
6	32	15	1480	< 2	3.4	12	proliferative	53	1000	endometrioma	ND	ND
16	25	14	115	< 2	1.1	1.0	proliferative	10	9130	peritoneal	ND	ND
22	45	15	—	—	—	—	—	—	—	endometrioma	40	—
24	23	13	230	3	5.0	7.5	proliferative	84	2050	endometrioma	14	700
Luteal phase												
4	32	19	680	> 32	0.7	0.7	secretory	10	2745	endometrioma	ND	—
5	42	20	—	—	—	—	secretory	8	—	endometrioma	8	—
13	34	19	600	26	0.6	0.9	secretory	ND	2740	ovarian	ND	—
7	18	30	240	4.4	1.5	2.7	secretory	47	—	endometrioma	ND	ND
8	27	23	460	12	0.8	1.4	—	—	—	cicatrice	6	ND
9	46	22	—	—	—	—	—	—	—	endometriosis	—	—
11	35	22	—	—	—	—	—	—	—	endometrioma	ND	ND
20	43	26	340	8.4	1.0	< 0.5	secretory	9	2000	endometrioma	ND	—
23	44	20	490	14	0.8	0.5	secretory	14	363	endometrioma	1	ND

ER levels were assayed by isoelectric focusing of cytosol on polyacrylamide gel after trypsinization *ad modum* Gustavsson *et al.* (3).

The PR assay was performed by a dextran-coated charcoal (DCC) method. In some cases the endometriotic lesions were too small to obtain enough tissue for the PR assay.

All laboratory procedures were performed at 0–4°C, unless otherwise mentioned.

The receptor concentrations were related to mg cytosol protein, determined according to Lowry (7).

Chemicals. [2,4,6,7- 3 H]-estradiol-17- β (spec. activity 91–108 Ci/mmol), Code TRK 322, was purchased from the Radiochemical Centre, Amersham, U.K., [6,7- 3 H]-R 5020 (17 α ,21-dimethyl-19nor-4,9-pregnandiene-3,20-dione, spec. activity 87 Ci/mmol, 98.5% purity) from New England Nuclear, Boston, Mass., U.S.A. and progesterone from Seraloids, Inc., Wilton, New Hampshire, U.S.A. Trypsin (E.C. 3.4.4.4.) from bovine pancreas, type III, was obtained from Sigma Chemical Company, St. Louis, Mo., U.S.A., polyacrylamide gel (Ampholine) PAG-plate, pH 3.5–9.5, from LKB Produkter AB, Sweden, and Luma gel from Lumac Systems AG, Basle, Switzerland.

Determination of cytosol ER. The frozen tissue specimen was cut into small pieces and 3 ml TE-buffer solution (10 mM Tris-HCl (pH 7.4), 1.5 mM disodium (EDTA) containing 7.5 nM of [3 H]estradiol), was added per gram tissue. After incubation for 30 min, the tissue was homogenized in 5-sec periods at 5-sec intervals, using a Polytrone homogenizer (Kinematica, Lucerne, Switzerland) until all tissue was homogenized. If the tissue sample was less than 0.15 g, a glass homogenizer was used instead. After further incubation for 45 min, the homogenate was centrifuged at 28000 $\times$ g for 10 min (Sorvall centrifuge SS-34). The super-

natant (cytosol) was kept at -20°C overnight and its protein content was then determined on an aliquot of the supernatant. CaCl₂ was added to the cytosol to a final concentration of 2 mM. To 300 μ l cytosol, 22 μ l trypsin per gram per liter protein was added and the mixture incubated for 30 min at 10°C. A half-volume was added of a DCC suspension (0.1% dextran, 1% charcoal in TE-buffer solution) to remove unbound estradiol. After incubation for 10 min the cytosol was centrifuged at 1700 $\times$ g for 10 min. 150 μ l of the supernatant was applied in plastic frames near the cathode on a polyacrylamide gel for isoelectric focusing for 1.5 hours at 50 mA, 30 W, with stepwise increased voltage to 1400 V. After focusing was completed the gel was cut in 3 mm broad slices and each slice was placed in a plastic vial. 10 ml Luma gel was added to the vial which was kept at 50°C for 1 hour and then shaken. The radioactivity was measured in a Wallac liquid scintillation counter 8100 (30% efficiency) for 10 min.

Determination of cytosol PR. The frozen tissue sample was cut into small pieces and 3 ml TE-buffer solution was added per gram tissue. Homogenization was performed in 5-sec periods at 5-sec intervals with a Polytrone homogenizer until all tissue was homogenized. Immediately after homogenization the homogenate was centrifuged at 28000 $\times$ g for 10 min (Sorvall centrifuge SS-34). Thereafter 100 μ l cytosol was mixed with 20 μ l tritiated promegestone ([6,7- 3 H]-R 5020) and 20 μ l unlabelled progesterone in buffer solution. The final concentration of [6,7- 3 H]-R 5020 in the buffer solution was 5 nM, while the final concentrations of unlabelled progesterone were 0, 3, 6, 9, 50 and 100 fold, respectively, molar excess to radiolabelled hormone.

After overnight incubation, free steroid was removed by adding 160 μ l DCC suspension and centrifugation at

Table IIa. Control group I. See Table I.

Case No.	Age	C. d.	Plasma				Endometrium		
			E ₂ pmol/l	P nmol/l	FSH µg/l	LH µg/l	Histologic picture	ER fmol/mg	PR prot
Follicular phase									
18	44	2	—	—	—	—	proliferative	109	—
4	48	5	700	< 2	4.6	0.7	proliferative	56	2400
12	30	26	—	—	—	—	proliferative	11	3800
13	53	7	610	< 2	7.6	12	proliferative	39	—
16	47	14	1780	2.0	8.2	8.4	proliferative	13	5920
17	29	14	210	< 2	2.2	> 22	proliferative	200	848
20	46	15	710	3.2	2.6	3.2	proliferative	56	1500
Luteal phase									
22	45	20	380	> 32	3.0	1.4	secretory	83	1400
26	32	—	370	27	2.6	2.0	secretory	ND	4728
29	45	—	—	—	—	—	secretory	30	3270
1	39	28	190	7.5	2.4	0.9	secretory	24	810
3	48	24	548	14	0.1	0.9	secretory	9	—
5	42	24	—	—	—	—	secretory	—	758
6	46	20	—	—	—	—	secretory	40	667
11	45	25	240	29	0.9	1.2	secretory	19	ND
14	45	33	880	25	2.1	1.8	secretory	5	4870
23	37	28	110	23	4.4	2.1	secretory	6	580

Table IIb. Control group II. See Table I.

Case No.	Age	C. d.	Plasma				Endometrium		
			E ₂ pmol/l	P nmol/l	FSH µg/l	LH µg/l	Histologic picture	ER fmol/mg	PR prot
Follicular phase									
14	41	14	380	< 2	4.1	17	proliferative	—	ND
6	49	12	1420	2.4	3.2	4.6	proliferative	12	3710
21	36	16	1500	3.4	1.0	2.2	proliferative	—	ND
8	43	10	1710	< 2	4.4	7.5	proliferative	7	2660
Luteal phase									
9	22	14	1100	32	2.8	2.5	secretory	—	867
10	41	16	250	26	2.5	4.4	secretory	56	1630
15	44	16	520	> 32	1.2	1.7	secretory	7	378
18	44	17	600	18	1.0	1.4	secretory	—	500
19	52	15	820	17	1.8	< 0.5	secretory	—	360
1	38	25	—	—	—	—	secretory	12	ND
11	41	34	410	> 32	1.2	0.5	secretory	9	—
12	42	27	445	7.6	1.9	2.9	secretory	—	370
22	52	28	140	3.8	0.7	0.9	secretory	18	—

1700 × g for 10 min. Ten milliliters of Luma gel was added to 150 µl cytosol and shaken vigorously. The radioactivity was determined for 2 min in the scintillation counter described above. The affinity constant and number of binding sites were calculated by Scatchard plot analysis (12).

RESULTS

Tables I and II give details of hormonal parameters, histological classification and ER and PR concentra-

tions of the individual cases. Figs. 1 and 2 show that the concentrations of ER and PR were markedly lower in the endometriotic tissue than in the endometrium itself. All endometrial samples but one contained measurable amounts of ER, while ER could be detected in only 6/20 endometriotic samples. All endometrial samples contained PR, while the corresponding figures for the endometriotic tissues were 2/9. The receptor concentration of the endometrium was the same in patients with endometriosis as in the

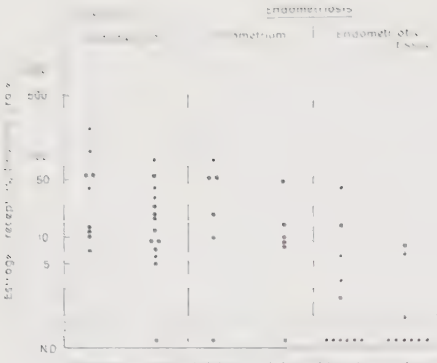


Fig. 1. Cytosol estrogen receptor concentration in endometrium and endometriotic tissue. Dashed horizontal line indicates the detection limit of the receptor assay.

control group. Fig. 3 illustrates a histogram from isoelectric focusing of ER on polyacrylamide gel and Figs. 4 and 5 illustrate the Scatchard analysis of the two tissue types from the same patient.

DISCUSSION

The relative levels of ER and PR found in endometrial cytosol during the follicular and luteal phases, respectively, were in agreement with previous reports (6, 8, 10, 13). In absolute terms our results are also similar to those reported previously with regard to the concentration of cytoplasmic ER in endometri-

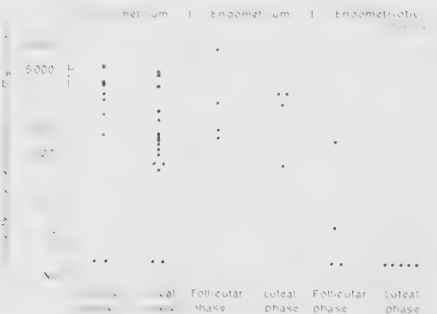


Fig. 2. Cytosol progesterone receptor concentration in endometrium and endometriotic tissue. Dashed horizontal line indicates the detection limit of the receptor assay.

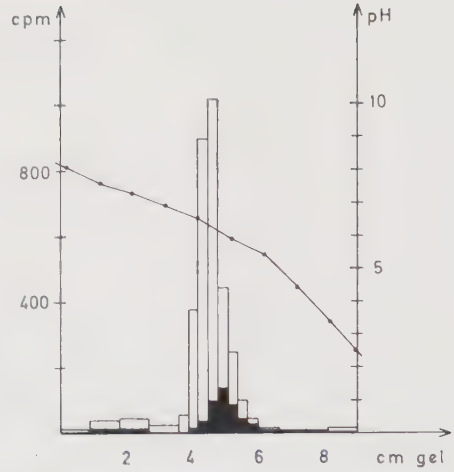


Fig. 3. Isoelectric focusing on polyacrylamide gel in the ER assay of cytosol from endometriotic tissue (endometrioma, case no. 24).

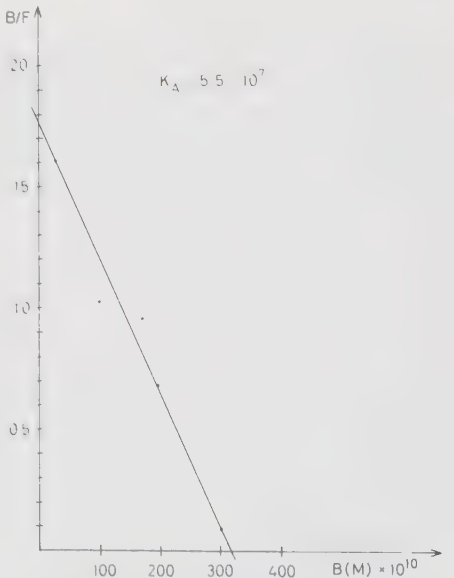


Fig. 4. Scatchard analysis of the progesterone receptor in cytosol from endometrium (case no. 24).

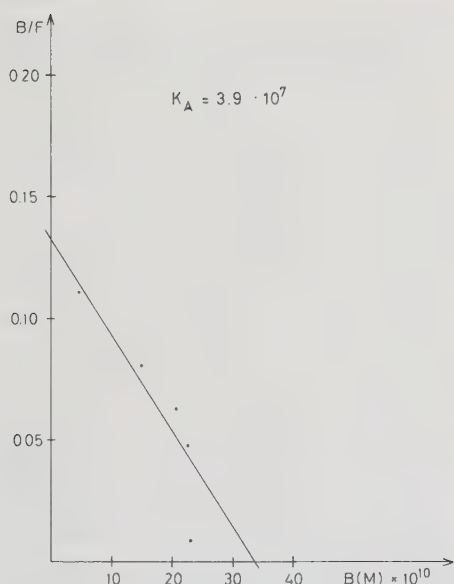


Fig. 5. Scatchard analysis of the progesterone receptor in cytosol from endometriotic tissue (case no. 24).

um, but we recorded a higher PR concentration in the endometrium than was found by most previous investigators. This may partly be due to differences in assay methods. In our procedure the radioligand was ³H-R 5020, while the unlabelled ligand was progesterone. When the combination of ³H-R 5020 and R 5020 was used instead, the receptor concentration was found to be about one-half to one-third of the levels obtained with the routine method used (unpublished data). This does not altogether explain the discrepancy between our PR concentrations and those reported by others. However, this discrepancy is of minor consequence, since the main purpose of the present study was to compare the relative receptor densities in endometrial vs. endometriotic tissue.

The finding of a relatively lower concentration of both ER and PR in endometriotic tissue compared with endometrium was not unexpected with regard to the discrepancies in histologic appearance of the two tissues. Nevertheless, the paucity of these receptors was rather surprising. While our study was in progress, two papers have been published which refer to cytosol receptor concentrations in endometriotic tissue. In 1979 Tamaya *et al.* (14) reported ER and PR

concentrations in 7 cases of ovarian endometriosis. The ER and PR contents in endometriotic tissue were substantially lower than in the corresponding endometrium. In particular, PR levels in endometriotic lesions were considerably lower (approximately one-fourth) although measurable quantities of PR were found in 6 out of 7 endometriotic samples. In 1980 Jänne *et al.* (5) reported that endometriosis was characterized by low or absent ER content but high PR content. The latter finding contrasts with the results obtained by Tamaya *et al.* and by ourselves.

In all three studies, the PR assay was performed by the DCC method, but different ligands were used.

The differences in receptor concentration in endometriotic tissue found in these three studies could be due to differences in tissue sampling or assay methods. In neither of the other two publications were any statements made regarding the dissection of the tissue samples. Therefore it is uncertain whether exclusively endometriotic tissue was assayed. Normal ovarian tissue has been shown to contain rather large quantities of specific progesterone (4, 9) as well as estrogen (2) binding macromolecules. As previously mentioned, in our study, histological examination of the tissue specimen taken from the endometriomas showed exclusively endometriotic tissue. The receptor characteristics reflected in the assay methods used were the same in endometriotic tissue as in endometrium. When isoelectrically focused, ER from the two tissues showed a peak with maximum at pH approx. 6.5. There was no significant difference in the binding characteristics of PR in the two types of tissue studied ($K_A = 4.5 \times 10^7 \pm 3.8 \times 10^7$ (mean $\pm$ SD) for endometrium and $K_A = 2.6 \times 10^7 \pm 1.6 \times 10^7$ for endometriotic tissue (Student's t-test, $p \geq 0.05$)). The assay method employed measures predominantly free receptors of estrogen and total (i.e. both free and occupied) receptor sites of progesterone in cytoplasm.

The finding of markedly lower concentrations of cytoplasmic ER and PR in the supposed ectopic endometrium suggests a reduced cellular responsiveness to hormone stimuli compared with intrauterine endometrium. The occurrence of such a difference is suggested by the histological signs of asynchronous reactions to hormonal influence. However, our study did not permit evaluation of other differences in the receptor function such as an increased nuclear receptor concentration or a diminished rate of receptor synthesis with subsequent determination of biologic response. A marked shift in the cytoplasmic/nuclear ratio of ER and PR immediately following ovulation

has been reported in normal endometrium, the concentration of nuclear receptors being at its highest at a time when the cytoplasmic concentration is decreasing sharply. In early pregnancy, most receptors are found to be intranuclear, only a very small amount being located in the cytoplasm (1, 6). In endometriotic tissue, a qualitatively similar event could well result in cytosol ER and PR concentrations falling below quantifiable levels.

In conclusion, current data on cytoplasmic ER in endometriotic tissue consistently show lower levels than in the corresponding normal endometrium. Data relating to concentrations of cytoplasmic PR in endometriotic tissue are conflicting and need further clarification. However, the differences in cytosol receptor concentrations indicate a functional discrepancy between endometriotic tissue and endometrium.

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Histochemical Localization of Estrogen and Progesterone Receptors:

Evaluation of a Method¹

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A histochemical method for the detection of estrogen (ER) and progesterone (PR) receptors in human endometrium, using estrogen and progesterone derivatives linked to fluorochrome-labeled bovine serum albumin (E₂-BSA-fluorescein isothiocyanate (FITC) and progesterone-BSA-tetramethylrhodamine isothiocyanate (TMRITC)), has been evaluated. The fluorochrome-labeled steroids were bound to the cytoplasm—preferably in glandular epithelial cells but to a lesser extent also to stromal cells. The steroid specificity of the observed binding was studied by preincubating the sections with a series of unlabeled steroids and nonsteroidal, hormonally active compounds (estradiol-17 β , diethylstilbestrol, tamoxifen, 5 α -dihydrotestosterone and R 1881 for ER and ORG 2058,

R 5020, dexamethasone, cortisol and 5 α -dihydrotestosterone for PR). The inhibition studies indicated that E₂-BSA-FITC and progesterone-BSA-TMRITC bind to ER and PR in human endometrium with a reasonable degree of specificity. The method was reproducible and various procedural steps were tested, showing satisfactory technical stability. The method is applicable to small tissue samples, and is a valuable complement to quantitative biochemical receptor assays, as it localizes the receptors in tissue slices.

KEY WORDS: Human endometrium; Estrogen receptor; Progesterone receptor; Histochemical steroid receptor detection; Fluorochrome-labeled steroids; Steroid binding specificity.

Introduction

Most techniques for quantitative steroid receptor assay are based on the specific binding of a radioactive steroid ligand to the receptor protein in a homogenized tissue sample (12). These methods usually require large tissue samples and do not yield any information about the distribution of the cells containing receptors, or about the distribution of the receptors within such cells (39). Certain ligand techniques, e.g., those using isoelectric focusing on polyacrylamide gel (IFPAG), have been successfully applied in estrogen receptor assay in biopsies obtained by fine needle aspiration (38). Such biopsies may contain more homogeneous cell populations, but the lesion must be readily accessible for puncture.

In recent years various histochemical methods that demonstrate steroid receptors in tissue sections have been de-

scribed (5,14,16,22,23,25,28,42). These methods allow the use of small tissue samples and provide information about the localization of the receptor containing cells. However, since these methods do not allow isolation or biochemical characterization of the steroid-receptor complex (e.g., the isoelectric focusing step in the IFPAG method and the determination of affinity constants), their validity has to be carefully evaluated by indirect means.

The present article concerns the evaluation of a method for histochemical localization of estrogen and progesterone receptors based upon fluorochrome-labeled steroids.

Materials and Methods

Tissues Samples

Samples of human endometrial tissue, mostly in late proliferative phase, were obtained by curettage or hysterectomy. Small pieces of the rectus abdominis muscle, obtained at laparotomy, were used as negative control tissue. Striated muscle has previously been shown to be devoid of estrogen receptor (ER) and progesterone receptor (PR) (6,18,25).

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One part of the endometrial sample was fixed in 4% neutral formalin, embedded in paraffin, processed for routine histologic examination, and dated basicaly according to Noyes et al. (27); a second part was frozen within 1 min in liquid propane cooled by liquid nitrogen for histochemical receptor detection; and a third part was frozen for biochemical receptor assay. The two specimens for receptor studies were stored, for no longer than 1 month, at -70°C until analyzed. Blood samples for serum hormone assay were collected during surgery.

Reagents

Bovine serum albumin (BSA), cortisol, diethylstilbestrol (DES), 5 α -dihydrotestosterone (5 α DHT), estradiol-17 β (E₂), and tetramethylrhodamine isothiocyanate (TMRITC) were obtained from Sigma Chemical Co. (St Louis, MO); fluorescein isothiocyanate (FITC) from Merck and Co., Inc. (Rahway, NJ); 16 α -ethyl-21-hydroxy-19 nor-4-pregnene-3,20-dione (ORG 2058) from The Radiochemical Centre (Amersham, Bucks., England); 17 β -hydroxy-17 α -methyl-4,9,11-estratrien-3-one (R 1881) and 17,21-dimethyl-19-nor-4,9-pregnan-3,20-dione (R 5020) from New England Nuclear (Dreieich, W. Germany). *Trans*-1-(4 β -diethylaminoethoxyphenyl)-1,2-diphenyl but-1-ene (tamoxifen) was kindly supplied by ICI-Pharma Ltd. (Gothenburg, Sweden) and Dexamethasone by Merck, Sharp and Dohme Research Laboratories, Merck and Co., Inc. (Rahway, NJ). BSA-FITC and BSA-TMRITC were prepared according to Lee (16).

Histochemical ER and PR Assay

The frozen specimens were cut in a cryostat and the unfixed sections, 6-8 μ m thick, were mounted on gelatin-coated slides. The histochemical method of Lee (16) was applied on consecutive sections, using estradiol-17 β -6-carboxymethyl oxime-bovine serum albumin-fluorescein isothiocyanate (E₂-BSA-FITC) for the ER localization and progesterone-11 α -hemisuccinate-bovine serum albumin-tetramethylrhodamine isothiocyanate (progesterone-BSA-TMRITC) for the PR localization (Fluoro-Cep-estrogen and Fluoro-Cep-progesterone, Zeus Scientific, Inc., Raritan, NJ).

Test procedure. The frozen sections were air dried at +4°C for 1 hr and then rehydrated for a few seconds with 2% BSA in phosphate buffered saline (PBS), pH 7.4. Excess buffer was wiped off and the sections were covered with 0.1 ml of Fluoro-Cep-estrogen or Fluoro-Cep-progesterone reagent. The slides were incubated in a moist chamber that was placed on a slow motion cradle for 2 hr at room temperature. Excess conjugate was drained off and the slides were immersed twice in PBS, pH 7.4, for 30 min in each bath. Cover slips were mounted in buffered glycerol and cemented to the glass to prevent the slice from air drying. For the microscopic examination, an Orthoplan fluorosence microscope (Leitz, Wetzlar) was used, equipped with a Ploem illuminator and a mercury vapor lamp (CS200W-4, Philips); for FITC, a 460-485 nm (B₂-Blue) excitation filter and a K 530 barrier filter were used, and for TMRITC, a 535-550 nm (G-Green) excitation filter and a K 580 barrier filter were used. The sections were examined within 24 hr by two independent viewers (A.B., O.L.) with no prior knowledge of the type of test procedure or blocking experiment followed. All slices were examined at least three times. The intensity and binding pattern of the fluorescent substance were classified in four groups as listed in Table 1. A consecutive section from each specimen was fixed in 4% formalin and stained with hematoxylin and eosin for histological identification. The histochemical examination was done before the biochemical receptor assay results were known.

Table 1. Visual grading of the extension of bound fluorochrome-labeled steroid in the glandular portion of the endometrium		
Negative	-	no glands fluorescent
Weak positive	+	less than 10% of the glands fluorescent
Medium positive	++	more than 10% but less than 70% of the glands fluorescent
Strong positive	+++	more than 70% of the glands fluorescent

Variations of procedural steps. With E₂-BSA-FITC as reagent the following procedural steps were evaluated: the time during which the tissue specimen was kept at room temperature before freezing in propane/liquid nitrogen (1, 10, and 30 min); the length of the drying period before incubation (5, 10, 20, 30, 60, and 120 min); the storage temperatures of the tissue before incubation (+4 and +23°C); the time of preincubation with blocking substances (30, 90, and 120 min); and the media used for rehydration of the tissue (PBS, pH 7.4, both with and without 2% (w/v) BSA). For each step evaluated the same tissue sample was used.

Studies on the homogeneity of the steroid-fluorescent derivatives. Two lots of E₂-BSA-FITC and one lot of progesterone-BSA-TMRITC were analyzed by gel filtration or subjected to dialysis. Gel filtration was performed in Sephadex G-25 medium (PD-10 prepacded columns, Pharmacia Fine Chemicals, Uppsala, Sweden) in PBS, pH 7.4. The relative fluorescence of the eluate fractions was determined using a JASCO FP-4 fluorescence spectrophotometer. The eluate fractions from the gel filtration of E₂-BSA-FITC were analyzed for immunoreactive E₂ equivalents by radioimmunoassay (19). A similar attempt to determine immunoreactive progesterone equivalents was unsuccessful, since they did not bind to any of the three different progesterone antibodies available at our laboratories.

Dialysis was done using a cellulose dialysis bag (Union Carbide, Chicago, IL) with a porosity of 24 Å. Two hundred microliters, both of the original E₂-BSA-FITC and of progesterone-BSA-TMRITC, were dialyzed against 1000 ml of 0.05 M sodium phosphate buffer, pH 7.4, at +4°C for 48 hr.

The eluate fractions with the highest concentrations of steroid-BSA conjugates from the gel filtration and the dialyzed conjugates were thereafter tested in tissue incubations as described above.

Studies on hormone binding specificity. Prior to the addition of the fluorescent steroid-albumin conjugates, consecutive sections of endometrial tissue were preincubated for 90 min at +4°C or room temperature with different steroids and nonsteroidal, hormonally active compounds as listed in Tables 2 and 3. All solutions of these compounds were prepared by diluting stock solutions in ethanol with PBS, pH 7.4. Before dilution with PBS, the tamoxifen base in the stock solution was neutralized with a stoichiometric amount of 1 M HCl. Because of the low solubility of some steroids in the buffer, no molar excess of the blocking substances could be obtained and the ethanol concentration had to be high. The final ethanol concentration was 1 or 10% (v/v); appropriate controls on consecutive sections were run with the same ethanol concentrations in PBS without steroid.

In the blocking experiments, several of the preincubations were performed on each tissue sample, with every 4th to 6th slice serving as a control without preceding preincubation. In these studies a total of three lots of each Fluoro-Cep conjugate was tested, and each type of blocking experiment was performed for each lot. Incubation only

with either FITC-BSA or TMRITC-BSA was performed to study whether the presence of the steroid part of the conjugate was necessary for the binding. Inhibition was also studied by mixing a tamoxifen solution, 2.5×10^{-6} M, with the E₂-BSA-FITC reagent and by mixing an ORG 2058 solution, 2.2×10^{-7} M, with the progesterone-BSA-TMRITC reagent. All blocking experiments were performed on at least two different occasions on slices from the same tissue sample in order to check reproducibility of the method. The method was repeated on tissue samples from different patients to check the validity of the binding pattern.

Quantitative Determination of Cytosol ER and PR by Radioactive Ligand Techniques

ER determination was performed by IFPAG following limited trypsin digestion as described by Gustafsson et al. (9) and with [2,4,6,7-³H] E₂ as radioactive ligand. Determination of PR was carried out by a modification (1) of the procedure described by Daehnfeldt et al. (4) using ORG 2058 as radioactive ligand, dextrane-coated charcoal (DCC) separation, and Scatchard plot analysis (37). ER and PR values are expressed as femtomoles (fmoles) of bound tritiated steroid per milligram of cytosol protein as determined according to Lowry et al. (21).

Hormone Assays

Serum levels of E₂ and progesterone were determined using radioimmunochemical techniques described previously (40).

Results

Binding of the Fluorochrome-labeled Steroid-Albumin Conjugate to Endometrial Tissue

With one exception (see Table 4) all endometrial slices that had not been exposed to blocking substances showed binding of the steroid conjugate and the same cells were mostly found to bind both E₂-BSA-FITC and progesterone-BSA-TMRITC in consecutive sections. The specific fluorescence was preferably localized to the cytoplasm of the glandular epithelial cells, but a weak binding was also seen in the stroma (Figure 1).

Negative Controls

No specific binding was found in the striated muscle samples and preincubation with Tamoxifen or ORG 2058 did not change the weak background fluorescence.

Variation of Procedural Steps

In some tissue samples that had been kept at room temperature for 10 or 30 min before freezing, fewer glands were fluorescent compared to the part of the tissue sample that had been frozen immediately. Storage of the specimens dry at room temperature for more than 60 min before incubation sometimes resulted in lysis of the tissue and loss of binding capacity. The blocking effect of tamoxifen upon the binding of E₂-BSA-FITC was more pronounced after 90 min of preincubation than after 30 min. Apart from this, variation of the different procedural

steps yielded no differences with respect to binding of the fluorescent steroid derivatives to endometrial samples and to negative controls.

Homogeneity of the Reagents

The results from the gel filtration analysis are given in Figure 2. E₂-BSA-FITC appeared as a sharp, highly fluorescent peak between 2.0 and 5.4 ml of eluate. A broader peak of weak fluorescence subsequently appeared between 11.0 and 44.6 ml of eluate. This peak contained low molecular components constituting 71% of the total fluorescence eluted from the column and its content of immunoreactive E₂ equivalents corresponded to 87% of the total amount. Progesterone-BSA-TMRITC showed a higher degree of homogeneity in the gel filtration; the steroid-protein conjugate appeared as a distinct fluorescent peak between 2.1 and 5.3 ml of eluate, followed by a very weak fluorescence between 6 and 38 ml. This amount constituted 18% of the total fluorescence eluted from the column.

When gel filtrated or dialyzed conjugates were compared with untreated ones in simultaneous incubations using the same endometrial tissue sample, the specific fluorescence was more extensive and more intense when the purified conjugates were used.

Specificity of Binding

The results from the inhibition studies are given in Tables 2 and 3. Ethanol up to 10% (v/v) did not affect the binding of E₂-BSA-FITC or progesterone-BSA-TMRITC to the endometrium and there were no signs of tissue destruction. In the blocking experiments the inhibition of binding was usually complete, but in a few experiments, it was incomplete with either very weak residual fluorescence in the glandular cells, or a few fluorescent glands scattered among a majority of nonfluorescent glands. In all experiments, however, there was a pronounced difference in the extent of the binding of the fluorescence between blocked and unblocked sections. Incubation with only FITC-BSA or TMRITC-BSA yielded no specific fluorescence.

Comparison between Histochemical Receptor Localization, Biochemical Receptor Assay, and Menstrual Cycle Phase

The data from the comparison between results obtained by histochemical and biochemical receptor analyses are given in Table 4, together with serum hormone levels from the time of tissue sampling and histological dating of the endometrial samples. No consistent correlations could be found between histochemical findings and biochemical assay results.

Discussion

The histochemical technique for ER and PR demonstration tested in the present work seems to have a satisfactory stability, since variation of the procedural steps did not noticeably affect

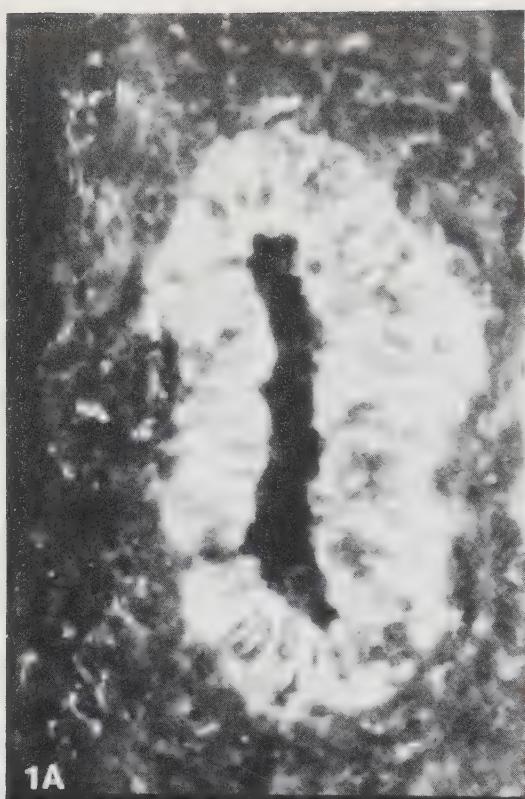


Figure 1. Fluorescence micrographs of two sections of endometrial tissue incubated with E_2 -BSA-FITC (A) and progesterone-BSA-TMRITC (B), respectively. The fluorescent hormone is specifically bound preferentially to the glandular epithelial cells but to a less extent also to the stromal cells. Original magnification $\times 588$.

the results. The gel filtration studies revealed a less satisfactory purity of the reagents. This is especially noticeable for the E_2 -BSA-FITC preparation. A high amount of impurities will result in a less sensitive method owing to lower specific fluorescence, and may also give a higher unspecific background fluorescence. The steroids and fluorescent components are coupled to the protein via stable covalent bounds. One possible explanation for the presence of nonprotein bound steroid may be the tendency of 17β -estradiol-6-CMO to undergo spontaneous intermolecular esterification (18); or again it may be due to hydrolysis of the protein chain during storage.

The inability of BSA-FITC and BSA-TMRITC to bind specifically to epithelial cells shows that the steroid component of the molecule is necessary for the specific binding to epithelial cells. Inability of BSA-FITC to bind specifically has previously been shown by Lee (16).

When discussing binding specificity, four classes of steroid binding proteins must be considered: a) unspecific high capacity, low affinity proteins such as albumin; b) specific high capacity, low to medium affinity proteins such as the estradiol binding protein in the human pancreas (31); c) low capacity, high affinity serum steroid binding proteins (e.g., sex hormone binding globulin (SHBG), corticosteroid binding globulin (CBG)) and d) steroid receptors (also low capacity high affinity proteins). Binding to groups a and b is excluded for following reasons: The binding of the fluorescent steroid-albumin conjugates to endometrial tissue is saturable, as indicated by the competition experiments. Furthermore, the fluorescence is specifically bound to epithelial and to a lesser degree to stromal cells. Finally, there is no specific binding to the abdominal muscle tissue, which would have been the case if the binding was due to unspecific transport proteins.

Contamination with steroid binding globulins (group c) from serum is sometimes a source of error in steroid receptor assays. The inhibitory effect of 5α DHT on the binding of E_2 -BSA-FITC might well be thought to indicate binding to SHBG (10,15,36). However, since tamoxifen and DES are known to bind only very weakly to SHBG (33), their pro-

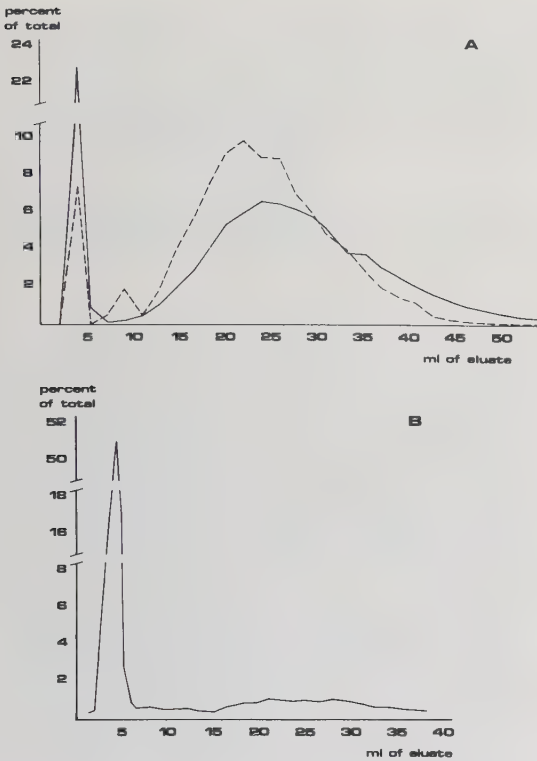


Figure 2. Relative fluorescence intensity and amount of immunoreactive steroid equivalents in consecutive portions of eluates from gel filtration of E₂-BSA-FITC (A) and of progesterone-BSA-TMRITC (B), through Sephadex G-25 (medium). The values are expressed as per cent of the total sum of all fractions. The dashed line indicates the amount of immunoreactive estradiol-17β equivalents and the solid line represents the fluorescence intensity at 492 nm (FITC) and 547 nm (TMRITC).

nounced inhibition of the binding of E₂-BSA-FITC clearly does not support this interpretation. Similar arguments probably apply to the binding of progesterone-BSA-TMRITC. The binding of progesterone-BSA-TMRITC was markedly inhibited by the synthetic gestagens ORG 2058 and R 5020,

both of which bind specifically to PR but not, or to a very low degree, to plasma proteins (11,20,34); thus CBG or SHBG can not have been responsible for the binding of progesterone-BSA-TMRITC. Furthermore, a high concentration of cortisol (1 × 10⁻⁴ M) only partially inhibited the binding to endometrial cells; had this binding been due to CBG contamination, it would have been completely inhibited. Finally, as mentioned above, the specific binding to epithelial cells is clearly incompatible with the binding of E₂-BSA-FITC and progesterone-BSA-TMRITC to endometrial samples being the result of any contamination with serum steroid binding globulins.

Cross-reactions between steroids and their respective receptors are well known (7,32,34). Since the synthetic androgen R 1881 binds specifically to androgen receptor (AR) but not to ER or SHBG (2,32,34), and is unable to inhibit binding of E₂-BSA-FITC, AR can not be the protein responsible for this binding. The inability of dexamethasone to inhibit binding of progesterone-BSA-TMRITC, excludes the glucocorticoid receptor (CR) as responsible for this binding (8,20). AR is ruled out by the fact that a high 5αDHT concentration only partially inhibits the binding and a complete inhibition was obtained by R 5020, which has a low affinity to AR (34). The partial inhibition caused by cortisol and 5αDHT can probably be ascribed to a possible low affinity of progesterone-BSA-TMRITC to PR (13). The results of the inhibition studies are thus strong evidence that the binding of E₂-BSA-FITC and progesterone-BSA-TMRITC to epithelial and stromal cells of the human endometrium is due to ER and PR, respectively.

While DES competes strongly with E₂ at the ER, the affinity of tamoxifen to ER is very low ($K_d = 10^{-7}$ M) (3). However, tamoxifen at a 1 × 10⁻⁶ M concentration completely inhibited the binding of E₂-BSA-FITC, as did DES in the same concentration. This may be explained by a lower affinity of the fluorescent E₂ conjugate to ER when compared with E₂ (30) and a low molarity of conjugated steroid in the reagents. Another explanation of the sensitivity of the histochemical ER assay to blocking agents may be the presence of high amounts of nonfluorescent estradiol derivatives in the preparation. These arguments may also be valid for the inhibitory effect of 5αDHT on this binding.

Only the synthetic gestagen R 5020 caused complete inhibition of the binding of progesterone-BSA-TMRITC. The inability of ORG 2058 to completely inhibit binding, even at

Table 2. Effects of steroids and other compounds upon the binding of E₂-BSA-FITC to human endometrium

Compound	Final concentration (M)	Ethanol (%; v/v)	Effect on binding of E ₂ -BSA-FITC
Estradiol-17β	1.1 × 10 ⁻⁵	10	complete inhibition
Estradiol-17β	1.1 × 10 ⁻⁶	1	pronounced but incomplete inhibition
Diethylstilbestrol	1.1 × 10 ⁻⁶	1	complete inhibition
Tamoxifen	2.5 × 10 ⁻⁶	1	complete inhibition
R 1881	4.1 × 10 ⁻⁴	1	no inhibition
5α-Dihydrotestosterone	1.1 × 10 ⁻⁴	10	pronounced but incomplete inhibition

Table 3. *Effects of steroids and other compounds upon the binding of progesterone-BSA-TMRITC to human endometrium*

Compound	Final concentration (M)	Ethanol (%; v/v)	Effect on binding of progesterone-BSA-TMRITC
ORG 2058	1.0×10^{-5}	10	pronounced but incomplete inhibition
R 5020	1.0×10^{-5}	10	complete inhibition
Dexamethasone	3.9×10^{-3}	10	no inhibition
Cortisol	1.0×10^{-4}	10	pronounced but incomplete inhibition
5 α -Dihydrotestosterone	1.1×10^{-4}	10	pronounced but incomplete inhibition

high concentrations, may be explained by the higher rate of dissociation of the steroid-receptor complex for this compound compared to R 5020 (13). Furthermore, in contrast to other compounds tested, the capacity of ORG 2058 to inhibit binding of [3 H]-progesterone to PR is independent of concentration between 10^{-8} – 10^{-6} M (13).

The fluorescent hormone was found preferentially in the epithelial cytoplasm. The ER and PR values found (using the IFPAG and the DCC techniques, respectively) were not very consistent with the cytochemical results. This type of comparison is hazardous, however, since the histochemical technique localizes minute quantities of receptor containing cells in a tiny tissue sample, whereas the biochemical technique determines the total amount of free steroid receptors in a relatively large homogenized sample. With biochemical techniques, receptors derived from a small number of receptor bearing cells scattered throughout a large tissue sample may be too few to be detected. Earlier studies have shown that the steroid-receptor content in the endometrium varies in dif-

ferent parts of the uterine cavity and at different depths of the endometrium, being highest in the fundal part and lowest in cervix (35,41). Since the specimens used for histochemical and biochemical analyses may be derived from different parts of the endometrium the concentration and distribution of the receptors may be different in the two samples. Lee (17) found no correlation between the biochemical and the histochemical results and Pertschuk et al. (29) concluded that the histochemical method appears to be more sensitive than the biochemical assay.

The histochemical method used is unsuitable for a quantitative determination of the free receptors. Uneven dispersion of the fluorophores, variation in section thickness, and variation in the amount of free steroid available in the reagents are some important errors that must be taken in account. Furthermore, the macromolecular conjugate of fluorochrome-BSA-steroid might be more or less hindered by its size from reaching all parts of the cell and thus may not reveal the total amount of free receptors (24,26).

Table 4. *Relationship between results of biochemical (IFPAG for ER and DCC for PR) and histochemical [using E₂-BSA-FITC (FC-E) and progesterone-BSA-TMRITC (FC-P)] steroid receptor analyses in 12 endometrial samples in which the cycle phase had been histologically determined^a*

Patient	Endometrial cycle phase ^b	E ₂ (pmol/liter)	ER ^c (fmol/mg protein)	FC-E	P (nmol/liter)	PR ^d (fmol/mg protein)	FC-P
1. B.H.	EP	250	11	+	3.4	1310	++
2. G.-B.H.	LP	930	44	—	4.1	1100	+
3. B.M.	LP	1540	3	+++	2.0	1450	++
4. M.N.	LP	1620	87	++	2.0	720	++
5. A.A.	ES	700	12	+++	27.6	440	++
6. S.F.	ES	600	31	+	25.1	555	++
7. E.L.	ES	550	20	+	4.0	600	+
8. I.L.	ES	330	50	+++	6.0	850	+++
9. B.O.	ES	580	11	+++	31.4	700	+++
10. I.L.	LS	320	2	+	5.0	680	+
11. I.-B.N.	LS	140	149	+++	4.9	1600	+++
12. M.W.	LS	570	60	++	10.9	910	++

^aFor the grading of the histochemical results see Table 1. Serum level of estradiol (E₂) and progesterone (P) at the time for tissue sampling are shown.

^bEP, early proliferative; LP, late proliferative; ES, early secretory; LS, late secretory.

^cLower detection limit 2 fmol/mg cytosol protein.

^dLower detection limit 50 fmol/mg cytosol protein.

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BINDING OF ESTROGEN AND PROGESTERONE TO HUMAN ENDOMETRIUM
IN THE DIFFERENT PHASES OF THE MENSTRUAL CYCLE. A HISTO-
CHEMICAL STUDY

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ABSTRACT

Specific estrogen and progesterone binding in human endometrium was studied histochemically using fluorochrome labeled steroids (estradiol-17 β -BSA-FITC and progesterone-BSA-TMRITC), endometrial samples from 36 women being investigated. The binding pattern was similar with both reagents. The relationship between the bindings to glands and to stroma, however, varied with the menstrual phase of the tissue. The specific fluorescence was more intense in the epithelial structures in the proliferative phase. In the secretory phase, the fluorescence from stromal cells was as intense as, or more intense than, that from the glands. The localization of the fluorophores in the glandular epithelial cells also varied by menstrual phase. In the proliferative phase, the fluorescence was most intense in the basal part or the whole cytoplasm of the glandular epithelial cells, while in the secretory phase the fluorescence was most intense in the apical and sometimes also in the basal part of the epithelial cells.

Key words: estrogen, progesterone, endometrium, histochemistry of steroid binding, menstrual phases.

INTRODUCTION

Estrogen and progesterone cytosolic receptors (ERc and PRc) in human endometrium have been demonstrated in several studies with biochemical assay techniques. The maximal concentration of ERc appears in the mid or late proliferative phase, and that of PRc in the late proliferative or early secretory phase (5, 11, 18, 21-23, 29, 31, 33-35, 38, 40). Some published results, however, have been conflicting (13, 30).

On the other hand, only a few histochemical studies on specific steroid binding in endometrium have been reported. Barrows et al. (1), found that both glands and stroma in the human proliferative endometrium stained strongly with fluoresceinated estradiol, while secretory endometrium revealed little glandular staining. A cyclical variation of the estrogen and progesterone binding pattern has also been found in the rat uterus (17). Using an autoradiographic method, Tchernitchin et al., (36), were not able to find any qualitative differences in the distribution of tritium labeled estrogen and progesterone bound in human endometrium at different phases.

The aim of the present investigation was to study possible variations in the binding of fluorochrome labeled estradiol and progesterone in human endometrial glands and stroma during the menstrual cycle.

MATERIAL AND METHODS

Endometrial specimens from 36 women (aged 40 ± 8 (SD) years) were obtained by curettage (33 specimens) or hysterectomy (3 specimens). Eighteen women were operated on because of bleeding irregularities, 16 because of endometriosis and 2 because of cancer in the uterine cervix. Only samples with a normal histological appearance

were included. No woman had been on hormonal medication in recent months.

The date for the last bleeding was noted and venous blood samples were drawn on the morning of the operation day, or on the following morning, and the serum concentrations of estradiol-17 β (E₂), progesterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH) were assayed radioimmunologically (37).

The tissue samples were immediately rinsed in isotonic saline at +4°C to remove blood and mucus, and then divided into three parts. One part, about 1 - 3 mm³ in size, was frozen in liquid propane cooled by liquid nitrogen within one minute and then stored at -70°C until required for the histochemical procedure. The second part was immediately placed in a dry plastic bag, stored at -70°C, and used for biochemical steroid receptor assays within one month. The third part of the tissue sample was fixed in 10% neutral formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin for histological dating according to Noyes et al. (24). The cycle day corresponding to the dominating phase of the tissue specimens was recorded. A histological pattern corresponding to cycle day 3-9 in a twenty-eight-day cycle was classified as early proliferative, day 10-15 as late proliferative, day 16-19 as early secretory, day 20-22 as mid secretory, day 23-27 as late secretory and day 28-2 as premenstrual and menstrual endometrium.

Both estradiol-17 β -6-carboxymethyl oxime-bovine serum albumin-fluorescein isothiocyanate (E₂-BSA-FITC), and progesterone-11 α hemisuccinate-bovine serum albumin-tetramethylrhodamine isothiocyanate (progesterone-BSA-TMRITC) FLUORO-CEPTM (Estrogen) and FLUORO-CEPTM (Progesterone), Zeus Scientific Inc., Raritan, N.J., USA, as described by Lee (15) were used for the histochemical localization of specific steroid

binding sites. The specificity of the steroid binding of the conjugate has been studied extensively (3) and been found to be acceptable. The specimens were handled in a strictly standardized way as described elsewhere (3). The frozen unfixed specimens were cut in a cryostat to 6-8 μ m thick sections. Two or three consecutive frozen sections were mounted on each gelatinized glass slide which was immediately placed in a freezer at -70°C . When the cutting procedure was complete, the glass slides were placed at $+4^{\circ}\text{C}$ and the sections were air dried for one hour. After rehydration for a few seconds with 2% BSA in phosphate buffered saline (PBS), pH 7.4, excess buffer was wiped off and the sections were covered with 0.1 ml of the FLUORO-CEPTM reagent. One duplicate set of slides for ERC and one duplicate for PRC were always prepared for localization of the estrogen and progesterone binding sites. After incubation for two hours in a moist chamber placed on a slow motion cradle at room temperature, the slides were immersed twice in PBS, pH 7.4 for 30 min. in each bath. On a fifth slide, consecutive sections were fixed in 10 % neutral formalin for at least 15 minutes and stained with hematoxylin-eosin for histological identification. Only sections containing more than 10 well-preserved glands were included.

The sections were independently examined within 24 hours after the incubation by two of us (A.B., O.L.) according to a standardized schedule. The proportion of fluorescent glandular epithelial cells (none, <10%, >10% but <70%, >70%) and the distribution of the fluorochromes within the glandular epithelial cells (basally, apically, the whole cytoplasm) and the stroma was estimated. A Leitz Orthoplan fluorescence microscope was used, equipped with a Ploem illuminator and a HB0200 mercury lamp. An 460-485 nm excitation filter and a K530 barrier filter were used for FITC and a 535-550 nm excitation filter and a K580 barrier filter were used for TMRITC.

It was possible to estimate the relative proportion of fluorescent epithelial cells because the tissue emitted a weak unspecific fluorescence which permitted the identification of all epithelial structures. This was not possible, however, in the case of the stromal cells, so grading of specific stromal fluorescence was omitted.

The histochemical examination was performed before the results of the biochemical receptor assays or serum hormone concentrations were known.

The quantitative ERc assays were performed using isoelectric focusing on polyacrylamide gel (9), and the quantitative PRC assays were performed using a dextran coated charcoal (DCC) method originally described by Korenman (4, 14). The amount of receptors found was related to the amount of cytosol protein assayed according to Lowry et al. (19).

RESULTS

The number of cases with different histological phase pattern appear from Table I.

There was a good correspondence between the histological dating of the endometrium and the peripheral plasma hormone concentrations in all cases. The concentration of FSH was consistently within the normal range for premenopausal women.

The binding pattern was the same irrespective of whether the sample was obtained by curettage or hysterectomy. In most cases the extent and the site of fluorescence were similar with both conjugates.

The relationship between the binding of fluorophores to glands and to stroma varied with the menstrual phase of

the tissue. In the proliferative phase the fluorescence was more intense in the epithelial structures than in the stroma (Fig. 1). In the secretory phase the reverse pattern was found, the fluorescence from the stromal cells being as intense as or even more intense than that from the glands (Fig. 2).

The localization of the fluorophores in the glandular epithelial cells varied with menstrual phase. In the proliferative phase, when the glands were straight or slightly tortuous and narrow, a strong specific fluorescence was observed mainly in the basal parts of the epithelial cells or in their whole cytoplasm (Fig. 3). On the other hand, the tortuous glands found in the secretory phase, displayed a weaker specific fluorescence which was located apically and sometimes basally as well, whereas the central part of the cell body was less fluorescent (Fig. 4). Saw-toothed glands in late secretory and menstrual phase were always negative. In very early proliferative phase the epithelium had not yet become fluorescent and the stroma was still fluorescent (Fig. 5).

When present, the epithelium covering the endometrium was invariably positive, even in sections where the glandular epithelium was negative, and the binding was not influenced by the menstrual phase. When stromal fluorescence occurred, this appeared to be more intense in the upper part of functionalis than in the deeper layers of the endometrium.

The results of the biochemical assays are presented in Fig. 6. In 6 samples the tissue was too sparse to allow both types of biochemical receptor assays to be done, and only PRC was assayed. The highest values of ERC were found in late proliferative and early secretory phase, and those of PRC in late proliferative phase.

DISCUSSION

The present study showed that the distribution of the specific fluorescence within the cytoplasm of the glandular cells and its distribution between the glandular structures and the stroma varied during the menstrual cycle. In the proliferative phase both conjugates were detected preferably in the epithelial structures, often localized basally or to the whole cytoplasm of the cells, whereas the binding to stromal cells was less noticeable. This is compatible with the morphological appearance during this phase, when high mitotic activity in the epithelial cells denotes estrogen effect. During this phase the stromal cells are small with poorly developed organelles and scanty cytoplasm. Stromal cells in mitosis generally abound until in late proliferative phase (6). Nonetheless, electron microscopy does reveal a slow maturation of these cells, with gradual enlargement of their nuclei and development of cytoplasmic organelles. It may be that the priming of the stromal cells by estrogen has a rapid effect on cell replication but a slower effect on cytoplasmic differentiation (32). In the secretory phase, the distribution of the specific fluorescence tended to be reversed, in that specific fluorescence was often most pronounced in the stroma instead. During this phase mitotic activity is uncommon in the epithelial cells and the influence of progesterone induces the formation of vacuoles, which are located basally in early secretory phase, but move to the apical region of the cells during mid secretory phase. However, mitoses occur in the stroma when the predecidual cells, rich in cytoplasm, develop as an effect of the second estrogen surge (32). This may explain the more intense fluorescence seen in the stromal cells of the secretory endometrium.

The ERc and PRc levels found in this study corresponded to those previously reported (5, 11, 18, 21-23, 29, 31, 33-35, 38, 40).

In some studies histochemical semiquantitative evaluation of steroid binding sites has been compared to steroid receptor concentrations assayed by biochemical methods (2, 7, 8, 10, 12, 20, 25-28, 42). Results have been contradictory, however, and in our opinion such comparisons can be considered invalid for a number of reasons. Although specificity studies have shown (3, 16) that the histochemical method has a reasonable degree of ligand specificity, it is still not known whether the two methods demonstrate the same types of steroid binding. Even if this were the case, however, there remain several other objections to the validity of such comparison. The histochemical method visualizes the steroid binding in separate cells that might be unevenly disseminated throughout a tissue sample, while the biochemical method determines the quantity of steroid receptors from a tissue homogenate which also contains varying amounts of non-receptor bearing tissue components.

The distribution of the endometrial glands varies in different parts of the uterine cavity and also at different depths of the endometrium (6) and the steroid receptor concentration has been found to decrease stepwise from the fundal part to the cervix region (5, 31, 34, 39). Histochemically the extent of steroid binding has been found to differ between different areas of the endometrium, being commonly more extensive in the functionalis (41). Furthermore, in most tissue sections some glands differ from the majority of glands with respect to the menstrual phase pattern and also with respect to binding distribution of the fluorophores. This corresponds to morphological variations found in hematoxylin-eosin stained sections (6). Since samples taken for biochemical assay differ from

those used for histochemical examination, and may even derive from different parts of the uterine cavity this is a further important reason why the results of the two methods are not comparable from a quantitative point of view.

This, however, does not exclude the possible relevance of qualitative comparison. Our finding that there exists a cyclical covariation of the ERc and PRc levels, determined biochemically and of the extent and distribution of the specific steroid binding demonstrated histochemically, may be of physiological significance.

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TABLE I. Distribution of endometrial samples in relation to menstrual phase.

Phase	Cycle days	No. of samples
Early proliferative (EP)	3 - 9	2
Late proliferative (LP)	10 - 15	14
Early secretory (ES)	16 - 19	6
Mid-secretory (MS)	20 - 22	6
Late secretory (LS)	23 - 27	7
Premenstrual and menstrual (MP)	28 - 2	1

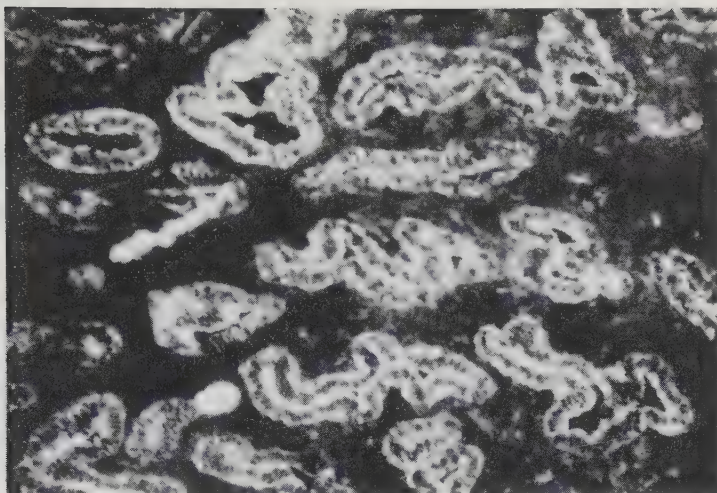


Fig. 1 Human endometrium in late proliferative phase. The whole glandular epithelial cells are fluorescent, the stromal cells are not fluorescent. Original magnification x 35.

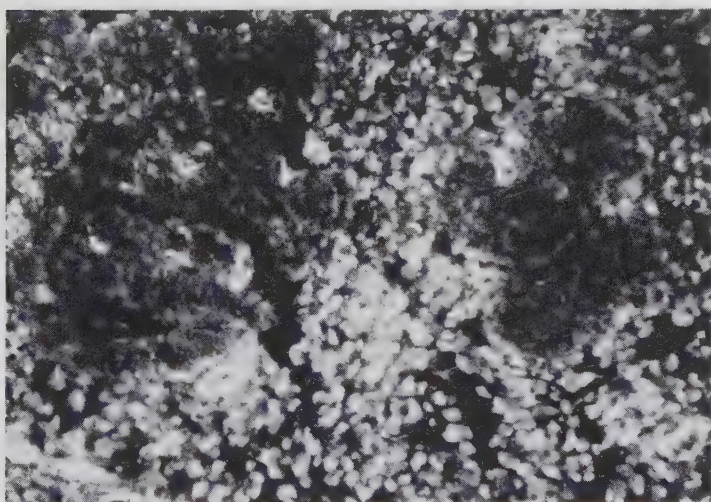


Fig. 2 Human endometrium in late secretory phase. The epithelial cells are not fluorescent while the stromal cells are fluorescent. Original magnification x 88.

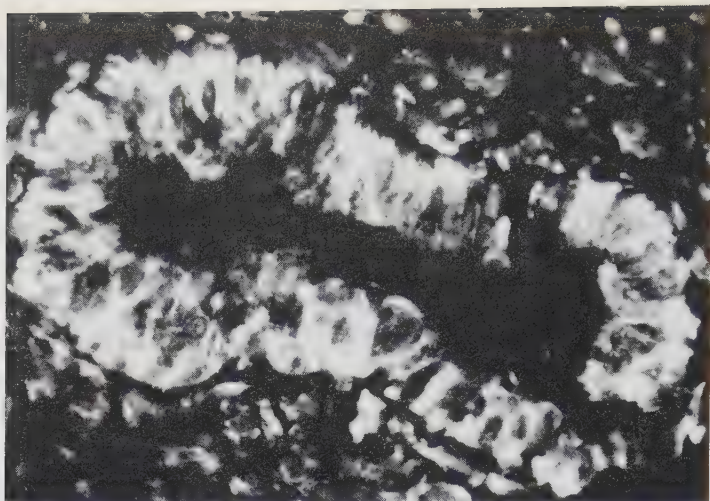


Fig. 3 Human endometrium in proliferative phase. The epithelial cells are fluorescent basally while the stromal cells are negative. Original magnification x 226.

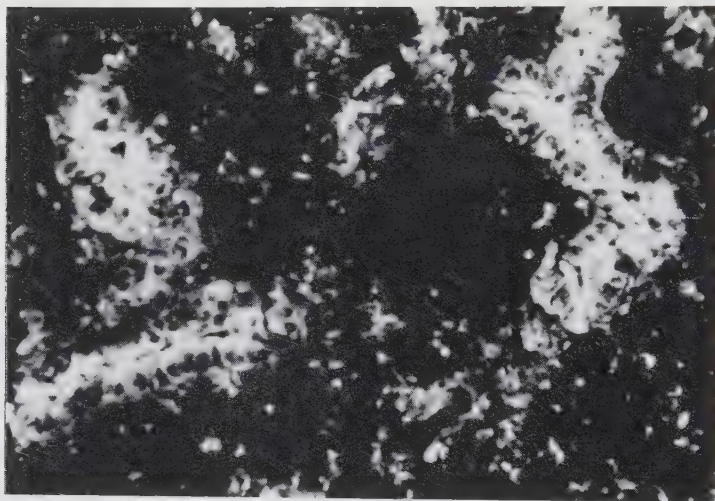


Fig. 4 Human endometrium in midsecretory phase. The epithelial cells are fluorescent apically and also the stromal cells are fluorescent. Original magnification x 35.

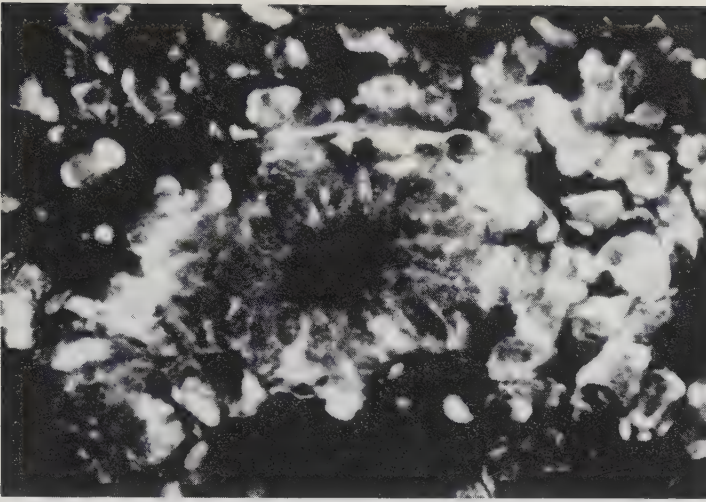


Fig. 5 Human endometrium in a) early proliferative phase. The great majority of the epithelial cells are non-fluorescent while most of the stromal cells are fluorescent. Original magnification x 226.



b) early proliferative phase. A very sparse fluorescence is found basally in the epithelial cells. Only a few stromal cells are fluorescent. Original magnification x 88.

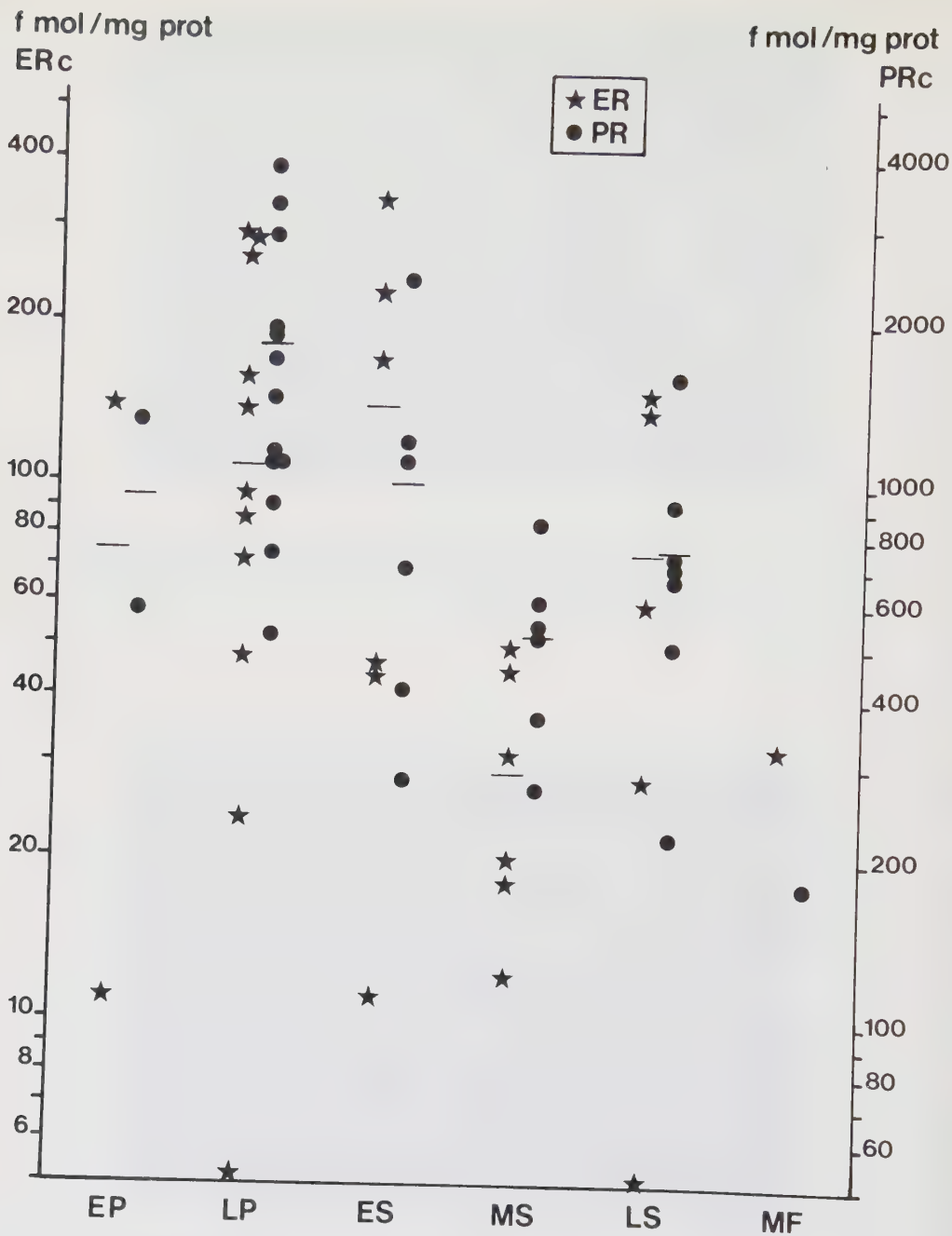


Fig. 6 Cytoplasmic estrogen and progesterone receptor levels biochemically assayed. (EP = early proliferative phase, LP = late proliferative, ES = early secretory, MS = mid-secretory, LS = late secretory, MF = menstrual phase, - mean value).

HISTOCHEMICAL DEMONSTRATION OF ESTROGEN AND PROGESTERONE BIND-
ING IN ENDOMETRIOTIC TISSUE AND IN UTERINE ENDOMETRIUM: A
COMPARATIVE STUDY

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Running headline: Steroid binding in endometriosis and
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ABSTRACT

Estrogen and progesterone binding to endometriotic and endometrial tissue was studied histochemically using estradiol and progesterone fluorochrome derivatives (E_2 -bovine serum albumin-fluorescein isothiocyanate and progesterone-bovine serum albumin-tetramethylrhodamine isothiocyanate). Thirty endometriotic samples from 21 women were studied, together with endometrial specimens obtained simultaneously from 14 of the women.

In 77% of the endometriotic samples binding of the estrogen conjugate was indicated by specific fluorescence in more than half of the epithelial cell population, and in 20% in less than half. The corresponding figures for the progesterone conjugate binding were 75% and 18%, respectively. Blocking studies indicated a reasonable degree of ligand specificity.

In endometrial tissue the corresponding figures were 64% and 29%, respectively, for binding of the estrogen conjugate and 54% and 38%, respectively, for binding of the progesterone conjugate. In 7 of 13 cases where evaluable samples of both tissues had been obtained, the relative proportion of fluorescent cells, with either reagent, was similar in the two tissue types.

Our results suggest that the cytoplasm of epithelial cells in endometriotic tissue and in uterine endometrium contains specific binding sites for both estrogen and progesterone. The binding pattern of the two conjugates in endometriotic tissue was unrelated to the menstrual phase.

Key words: Endometriosis; human endometrium; estrogen binding; progesterone binding; histochemical steroid receptor localization; fluorochrome-labeled steroids; steroid binding specificity.

INTRODUCTION

Although it is generally believed that endometriotic tissue and intra-uterine endometrium react similarly to hormonal influences, some studies have revealed certain histological (5, 8, 23) and ultrastructural (27) differences between the two types of tissue. A defect response to progesterone in endometriotic tissue, possibly due to a progesterone receptor deficiency, has also been suggested to be, at least partly, responsible for this structural discrepancy (8, 15, 23, 29).

The presence of hormone receptors is considered to be a prerequisite for hormonal response. Attempts at quantifying the cytosol receptors by means of biochemical assays have shown that, unlike the endometrium, endometriotic tissue has low, or even sometimes undetectable amounts of cytosol estrogen (ERc) and progesterone (PRc) receptors (2, 14, 29). However, biochemical assays require relatively large tissue samples, and since tissue samples from endometriotic lesions are often small and may contain only a few glands, the number of receptor-bearing cells may be too small to allow the receptors to be detected by these methods. Thus a histochemical method which visualizes the receptors within the cells, would be a very useful complement to biochemical assays.

So far there is no routine histochemical method capable of directly visualizing the receptor protein. However, several methods have been designed which may do so indirectly, by demonstrating binding of steroids to cells (9, 10, 11, 16, 17, 18, 22, 25, 30, 32).

Lee (18) has described a histochemical method using fluorochrome labeled estradiol and progesterone for localizing specific estrogen and progesterone binding sites. We have thoroughly evaluated this method and found that the fluorophores are bound to the cells with an acceptable ligand specificity (3).

The present communication describes the use of this technique to study the distribution of both estrogen and progesterone binding in endometriotic tissue and, where samples were available, to compare the binding pattern with that in uterine endometrial specimens obtained simultaneously from the same patients.

MATERIAL

Clinical material

Biopsies from endometriotic lesions were collected from 21 women, aged 36 ± 8 (mean $\pm$ SD) years. The patients had not received any medication during at least 6 months prior to the operation. Only samples containing glands or cysts with preserved lining epithelium and surrounded by specific stroma were included. In all, 30 samples of endometriotic tissue from varying anatomical sites were studied. In 14 cases only one sample was obtained, in 6 cases samples were derived from two lesions, and in one case from four different lesions. One sample from the ovary and one from the peritoneum were obtained in conjunction with laparoscopy, all other pelvic lesions were sampled during laparotomy. Twelve tissue samples were derived from small ovarian lesions, 11 from the inside of endometriotic cysts (by scraping with a scalpel), 3 from peritoneal lesions, 2 from scars in the abdominal wall, one from a vaginal lesion and, finally, one from a lesion on the serosa of the large bowel.

Endometrial samples, obtained by curettage, were taken simultaneously from the same woman in 14 cases.

Blood samples were collected on the morning of the operation day or the day after, and the serum concentrations of estradiol-17 β and progesterone were assayed radioimmunologically (31).

Chemicals

Estradiol-17 β (E₂), diethyl stilbestrol (DES), 5 α -dihydrotestosterone (5 α DHT) and cortisol were obtained from Sigma Chemical Co, St Louis, Mo, USA; 17 β -hydroxy-17 α -methyl-4,9,11-estratrien-3-one (methyltrienolone, R 1881) and 17,21-dimethyl-19-nor-4,9-pregnandiene-3,20-dione (promestone, R 5020) from New England Nuclear, Dreieich, W.Germany and 16 α -ethyl-21-hydroxy-19nor-4-pregnene-3,20-dione (ORG 2058) from The Radiochemical Centre, Amersham, Bucks., England. Trans-1-4 β -diethylaminoethoxyphenyl-1,2-diphenyl but-1-ene (Tamoxifen) was kindly supplied by ICI-Pharma Ltd., Gothenburg, Sweden, and dexamethasone by Merck, Sharp and Dohme Research Laboratories, Merck and Co., Inc., Rahway, N.J., U S A. Bovine serum albumin-fluorescein isothiocyanate (BSA-FITC) and bovine serum albumin-tetramethylrhodamine isothiocyanate (BSA-TMRITC) were prepared according to Lee (18).

For the histochemical localization of steroid binding proteins, estradiol-17 β -6-carboxymethyl oxime-bovine serum albumin-fluorescein isothiocyanate (E₂-BSA-FITC) and progesterone-11 α -hemisuccinate-bovine serum albumin-tetramethylrhodamine isothiocyanate (progesterone-BSA-TMRITC) (Fluoro-CepTM-estrogen and Fluoro-CepTM-progesterone, Zeus Scientific Inc., Raritan, N.J. USA) were used.

METHODS

The processing of endometriotic and endometrial samples for histochemical studies was performed as described previously for human endometrium (3, 4). Histochemical localisation of E₂ and progesterone binding was carried out according to Lee (18) using steroid-fluorochrome conjugates (E₂-BSA-FITC and progesterone-BSA-TMRITC, respectively). All sections were interpreted independently by two of us (A.B., O.L.).

The other part of the fresh endometrial specimens was fixed in 10% formalin, embedded in paraffin, sectioned and stained with hematoxylin-eosin to determine the menstrual phase as described by Noyes (24). Since in some cases the corresponding paraffin sections from endometriotic structures were too small to permit determination of the cycle phase, dating of endometriotic samples was abandoned.

The fraction of the total epithelial cell amount that showed specific fluorescence could be estimated (>50% or <50%) because a weak unspecific background fluorescence emitted from the tissue permitted the identification of all epithelial structures. We have also observed a specific fluorescence in endometrial stromal cells (4). Since the specific stroma in the endometriotic tissue samples, however, often showed some degree of bleeding and fibrosis, which could interfere with the specific fluorescence from the stromal cells, evaluation of the stromal fluorescence was abandoned in the present study.

The specificity of the binding of fluorochrome-labeled steroids to endometrium has been evaluated previously (3). The specificity of the binding to endometriotic tissue was analyzed in a series of competition studies. Prior to incubation with E_2 -BSA-FITC, consecutive sections were incubated for 90 min. at room temperature with the following substances: E_2 , DES, Tamoxifen, 5α DHT or the synthetic androgen R 1881 which has a high affinity for the androgen receptor. Similarly, before incubation with progesterone-BSA-TMRITC, consecutive sections were incubated with one of the following substances: ORG 2058 or R 5020, which are synthetic steroids with high affinity for the progesterone receptor, 5α DHT, dexamethasone or cortisol. In addition, sections were incubated with BSA-FITC or BSA-TMRITC only, to test unspecific binding of those compounds. The principles used for estimating the extent of the binding in the histological sections were the same as previously described for the endometrium (3).

RESULTS

The amounts of epithelial structures and the proportion of epithelial cells showing specific fluorescence in the endometriotic samples from different sites are given in Table I. Sections from three samples (two endometriotic and one endometrial) incubated with the fluorochrome labeled progesterone could not be evaluated for technical reasons. In 5 endometriotic samples the epithelial component consisted of parts of cysts or dilated glands, lined with preserved epithelium which in most cases displayed a strong specific fluorescence. The extent of the fluorescence was the same with both conjugates in all samples but three. There was no relationship between the extent of the fluorescence produced by the two conjugates on one hand and anatomical localization, surrounding fibrous adhesions, endometrial menstrual phase and serum hormone concentrations on the other.

All endometrial sections contained more than 10 glands. The menstrual phase of the endometrium as determined histologically is given in Table II. The serum concentrations of estradiol-17 β and progesterone corresponded well to the cycle phase determined histologically in the endometrium. Fluorescence indicating estrogen binding was found in more than 50% of the epithelial cells in 9 endometrial samples (64%), and in less than 50% in 4 samples (29%). No binding was found in one sample (7%). The corresponding figures for binding of the progesterone conjugate were 7 (54% of 13 evaluable samples) and 5 (38%) and the binding was absent in one sample (8%) (Table II). The extent of binding of the two conjugates in the endometrium was similar in 12 samples.

The binding patterns for the two conjugates in endometriotic tissue and endometrium are illustrated in figures 1 and 2. Specific fluorescence was demonstrated in the cytoplasm only. In the endometrium, the site of the specific fluorescence often varied, being either mostly basal or apical in the cells while

the whole cytoplasm of the endometriotic epithelial cells was usually more uniformly fluorescent. The binding of estrogen and progesterone conjugates in the endometriotic samples and in the endometrium in 14 cases is shown in Table II. The extent of the specific fluorescence with both conjugates in at least one endometriotic sample was similar to that in the endometrial sample from the same patient in 7 cases (54% of 13 samples). In 9 cases the estrogen conjugate was bound to the same extent in both tissue types; in 3 cases the specific fluorescence was more extensive in the endometriotic than in the endometrial tissue, and in 2 cases vice versa. The progesterone conjugate was bound to the same extent in both types of tissue in 7 cases, in 5 samples the binding of the progesterone conjugate was more extensive in the endometriotic than in the endometrial tissue, and in one case vice versa. In the 7 cases with differences in the extent of specific fluorescence, the discrepancy appeared to be unrelated to the menstrual phase histologically determined in the endometrial sample, and to the anatomical origin of the endometriotic sample.

The results of the blocking studies in endometriotic tissue (Table III) corresponded to a great extent with those previously found in endometrium (3). A slight difference was found after pre-incubation with tamoxifen, which resulted in a marked, though incomplete reduction of the specific fluorescence after incubation of the endometriotic samples with E_2 -BSA-FITC. The binding of progesterone-BSA-TMRITC to endometriotic structures was not inhibited by cortisol, but dexamethasone caused a weak inhibition. Sections incubated with BSA-FITC or BSA-TMRITC only did not display any specific fluorescence.

DISCUSSION

The present study showed E_2 -BSA-FITC and progesterone-BSA-TMRITC to be bound to the epithelial structures in most endometriotic samples. The binding of the estrogen conjugate in 97% of endometriotic samples, and of the progesterone conjugate in

93%, are in agreement with the results reported by Vihko et al. (33) who detected ERc in 70% and PRc in 94% of endometriotic tissue specimens, using a biochemical receptor assay technique. The levels of both ERc and PRc in endometriotic samples reported by these authors, were lower than those in the endometrial samples. The demonstration of binding sites for estrogen and progesterone may indicate a physiological basis for the treatment of endometriosis with gestagens or estrogen-gestagen combinations both of which have been used for many years (1, 12, 15 a.o.).

With the histochemical method used in our study, we were unable to find any variation in the binding pattern of the two conjugates in the endometriotic tissue, that could be related to the phase pattern in the corresponding endometrial sample. Using biochemical receptor assays, Vihko et al. (33), found that the ERc and PRc levels in endometriotic samples were significantly lower during the luteal than during the follicular phase of the cycle. In uterine endometrium, on the other hand, the topographic distribution of specific fluorescence with the two conjugates has been found to change according to the cycle phase (4). Cyclic variations in the amount of biochemically assayed cytosol steroid receptors have also been shown in several other studies (6, 13, 19, 20, 26, 28).

In 7 of 13 cases, the extent of the specific fluorescence from both conjugates was similar in both types of tissue. In 5 of the 6 cases where the extent of the binding of the progesterone conjugate in the two tissue types differed, the conjugate was bound to a higher extent in the endometriotic tissue than in the endometrium. Considering these findings, the postulated deficient response to progesterone in endometriotic tissue (8, 15, 23, 29) could hardly be explained by an inadequate binding of progesterone.

The blocking effect of different steroids on the binding of E₂-BSA-FITC was the same in endometrium and endometriotic

structures except for Tamoxifen. As this substance has a lower affinity ($K_d=10^{-7}$ M) to ERc than DES (7), the competitive effect may vary.

The blocking effect of dexamethasone and cortisol on the binding of progesterone-BSA-TMRITC differed somewhat in the two tissue types. In the endometrium dexamethasone did not inhibit the binding but in the endometriotic structures a weak inhibition was obtained. Cortisol caused a pronounced but incomplete inhibition of the binding in endometrium but no inhibition of the binding in endometriotic structures. Many studies have shown that glucocorticoids have a certain affinity for PRc (21). The affinity of dexamethasone is lower than that of R 5020 and that of cortisol is still lower (21). The differences found in the blocking studies between the two tissue types are difficult to evaluate and may not be significant.

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TABLE I

Distribution of samples according to amount of epithelial structures found in hematoxylin-eosin stained frozen sections and to the extent of specific fluorescence indicating estrogen (EB) and progesterone (PB) binding in the epithelial structures in 30 endometriotic samples from various anatomical sites.

Type of endometriotic tissue	cyst lining epithelium only	1-4 glands	>5 glands	++	EB +	-	++	PB* +	-
ovarian	1**	10	1	9	3	0	10	2	0
ovarian endometrioma***	4	5	2	7	3	1	7	2	2
peritoneal	0	3	0	3	0	0	2	0	0
vaginal	0	1	0	1	0	0	1	0	0
bowel	0	1	0	1	0	0	0	1	0
scar	0	1	1	2	0	0	1	0	0
Total number (per cent)	5(17%)	21(20%)	4(13%)	23(77%)	6(20%)	1(3%)	21(75%)	5(18%)	2(7%)

- *) not determined in two samples
- **) one cystic dilatated gland 3 mm in diameter
- ***) endometriotic cyst >3 cm i diameter
- ++) > 50% of the epithelial cells fluorescent
- +) < 50% of the epithelial cells fluorescent
-) no epithelial cells fluorescent

TABLE II. Binding of fluorochrome-labeled estradiol (EB) and progesterone (PB) to endometrium and endometriotic samples from 14 women taken simultaneously and related to menstrual phase.

E N D O M E T R I U M				E N D O M E T R I O S I S		
Pat.no.	Menstrual phase	EB	PB	Type	EB	PB
1	LP	-	-	ovarian*	+	+
2	LP	+	+	ovarian	+	+
				ovarian	+	+
3	LP	++	+	scar	++	++
4	LP	++	++	ovarian	++	++
5	ES	+	+	ovarian	++	++
				ovarian*	++	++
6	ES	++	++	ovarian	++	++
				ovarian	++	++
7	ES	++	++	ovarian	++	++
				ovarian	++	++
				bowel	++	+
				ovarian*	+	-
8	ES	+	+	vaginal	++	++
9	ES	+	+	ovarian	+	++
10	LS	++	++	ovarian*	++	++
				ovarian*	++	++
11	LS	++	++	ovarian*	+	+
12	DP	++	++	ovarian*	++	++
13	DP	++	++	peritoneal	++	++
14	DP	++	ND	ovarian*	-	-

* endometriotic cyst, > 3 cm in diameter

++ > 50% of the epithelial cells fluorescent

+ < 50% of the epithelial cells fluorescent

- no epithelial cells fluorescent

EP early proliferative (cycle day 3 - 9)

LP late proliferative (cycle day 10 - 15)

ES early secretory (cycle day 16 - 21)

LS late secretory (cycle day 22 - 27)

MP premenstrual + menstrual phase (cycle day 28 - 2)

ND not done

TABLE III

Results of competitive inhibition studies on the binding of E_2 -BSA-TMRITC and progesterone-BSA-TMRITC in endometriotic structures.

	Blocking substance	Concentration	Ethanol per cent	Degree of inhibition of specific fluorescence*
EB	Diethylstilbestrol	$1.1 \times 10^{-6} M$	1	complete
	Estradiol-17 β	$1.1 \times 10^{-6} M$	1	complete
	Tamoxifen	$2.5 \times 10^{-6} M$	1	pronounced but incomplete
	R 1881	$4.1 \times 10^{-4} M$	1	no inhibition
	5 α -dihydrotestosterone	$1.1 \times 10^{-4} M$	10	weak
PB	ORG 2058	$1.1 \times 10^{-5} M$	10	pronounced but incomplete
	R 5020	$1.0 \times 10^{-5} M$	10	complete
	Dexamethasone	$3.9 \times 10^{-3} M$	10	weak
	Cortisol	$1.0 \times 10^{-4} M$	10	no inhibition
	5 α -dihydrotestosterone	$1.1 \times 10^{-4} M$	10	pronounced but incomplete

++ fluorescence present in more than 70 % of the epithelial structures

+ in more than 10 % but less than 70 % of the epithelial structures and

- no fluorescence or fluorescence present in less than 10 % of the epithelial structures

* In sections not blocked with competitive substances, the fluorescence was present in more than 70 % of the epithelial structures (++) .

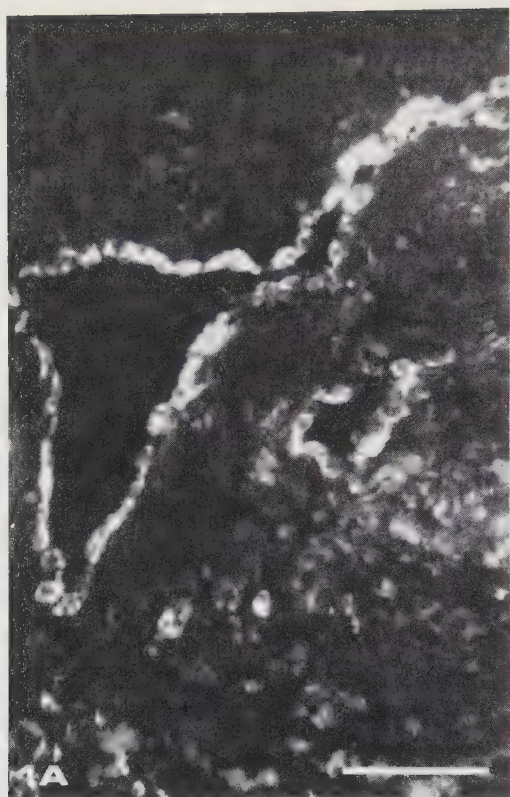


Fig. 1
 Frozen sections of endometriotic tissue incubated with
 E_2 -BSA-FITC (A) (original magnification $\times 226$) and
 progesterone-BSA-TMRITC (B) (original magnification $\times 88$).
 One bar is 100 μ m.



Fig. 2
Frozen sections of endometrium incubated with E_2 -BSA-FITC
(A) and progesterone-BSA-TMRITC (B). Original magnification
x 226. One bar is 100 μ m.

HUMAN ENDOMETRIUM TRANSPLANTED INTO NUDE MICE.

Histological effects of various steroid hormones.

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Running heading: Endometrial transplants in nude mice

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ABSTRACT

In a histological study of human endometrium transplanted into nude mice, eutopic endometrium from each of 8 women was transplanted subcutaneously to 4 nudes, one of which was treated with polyestradiol phosphate, one with medroxyprogesterone acetate, one with danazol, and one was left untreated as a control.

Grafts were removed after varying intervals of up to 57 days. Residual histologically evaluable endometrial tissue was found in 32 of the 96 grafts (33%). None of the extirpated grafts had the same histological pattern as the eutopic endometrium.

Besides confirming that human endometrium can be transplanted into nudes, our findings suggest that grafts react histologically in the same way as eutopic human endometrium to hormone treatment. The specific stroma seems to be crucial to a graft's capacity to respond to hormonal stimuli, and its replacement by fibrous tissue was accompanied by glandular epithelial changes very similar to those seen in human endometriotic lesions.

INTRODUCTION

Autologous transplantation of uterine endometrium has previously been performed on Rhesus monkeys, *Macaca mulatta* (1-4), rabbits (5-7) and rats (8) in order to elucidate the pathophysiology of endometriosis in vivo. However, in vivo studies of transplanted human endometrium have been impeded by immunological reactions, and only one study of human endometrium transplanted into nude mice has been reported previously (9).

The homozygous mouse mutant "nude" (nu/nu), has a congenital thymus aplasia (10,11) resulting in a deficient T-lymphocyte system (12-14). In addition, this animal has very low serum levels of some immunoglobulins (14-20). Therefore, such mice are suitable hosts for heterografts. The light microscopic (21-27) and ultrastructural appearance (28-30) of tissue grafts are generally identical to that of the primary tissue. Moreover, the chromosomal pattern has been shown to be unchanged (25,31,32), and biochemical properties to have been preserved (23,27-30,33-36). Thus, all the basic studies performed to characterize the human tissue/nude mouse system confirm that human tissue transplanted into nude mice retains human characteristics.

A wide variety of tumors (21-24,27-29,35-41) as well as normal human tissue (9,34,41-43) have been transplanted into nude mice. The taking rates of tumours have often been reported to be above 50% (21,24,25,27,28,32), whereas those of normal human tissue have often been lower (Furgyik, personal communication), and those of endocrine target tissues even lower still (35,40). Normal human tissue, except fetal tissue (34,43), does not grow expansively (9,41) in contrast to malignant tumour tissue.

Findings reported from extensive studies on the effect of various pharmacological substances on human tissue trans-

planted into nude mice (24,30,38,44) suggest that patterns of drug susceptibility are well preserved after transplantation.

The aim of the present study was to describe the histological appearance of normal human endometrium transplanted into nude mice, and to investigate whether histological changes could be induced by the administration of various hormonal compounds with known specific effects on normal human endometrium.

MATERIAL AND METHODS

Animals

Nude mice (nu/nu-BALB/c/AB spf qq) were supplied by Dr. C.W. Friis, Laboratory Animals Breeding and Research Centre, Gl. Bomholtgaard, Ry, Denmark. The animals were kept in open polypropylene cages measuring 325 x 205 x 115 mm, and covered by a stainless steel lid with compartments for food and water bottles. The cages were kept at 26-28°C in a room with limited access but without further specific pathogen-free conditions. The bedding consisted of aspen tree granulate (Torrax, ALAB Laboratorietjänst AB, Sollentuna, Sweden). The cages were changed weekly and cleaned manually. The diet consisted of pellets with mineral and vitamin additives (R3, Ewos-Anticimex, Södertälje, Sweden). Tap water was supplied *ad libitum* by means of a glass bottle with a conical top of stainless steel. All items of equipment were changed once weekly. The mice were 6-11 weeks old at the primary operation.

Tissue sampling

Specimens of human endometrium were obtained from 8 women (aged 30-53, mean 42 years) by curettage or at hysterectomy. Six women were regularly menstruating and the other two had irregular bleedings. The tissue samples were handled aseptically, and kept supplied with minerals and vitamins by being immersed immediately after collection in

sterile Petri-dishes containing Parkers medium 199 (SBL, Stockholm, Sweden).

One piece of each tissue specimen was fixed in 10% formalin, dehydrated and imbedded in paraffin. Sections, 6 μ m in thickness, were stained with hematoxylin-eosin for histological evaluation and dating according to Noyes et al (45).

Transplantation

The grafting procedure, performed in a laminar air flow bench, lasted for about 2 min. and was performed within 60 min. of the tissue specimen being collected. The mice were anesthetized with ether. In two cases 0.5 ml cloralhydrate (20 mg/ml) was given intraperitoneally. The tissue specimen was cut with a safety-razor blade into small pieces measuring about 1-2 mm³, three of which were implanted subcutaneously in the flank of each mouse via one cutaneous incision and subcutaneous debridation with a pair of blunt-ended scissors. The cutis was sutured with one stitch of Suturamide. Endometrium from each patient was transplanted to 4 mice. One recipient remained untreated while the others were injected subcutaneously with either of 3 steroid hormones: polyestradiol phosphate (Estradurin^R 80 mg/ml diluted with saline) 0.02 mg (0.001 mg/g b.w.), 0.1 ml every second week; medroxyprogesterone acetate (MPA, Depo-Provera^R 50 mg/ml) 5 mg, (0.25 mg/g b.w.), 0.1 ml weekly or danazol (17 α -Pregn-4-en-20-yno [2,3-d] isoxazol-17-ol) 4 mg (0.2 mg/g b.w.), dissolved in 0.1 ml of a sterile mixture containing 44% peanut oil, 46% benzyl bensoate and 10% phenyl carbinol given daily. The hormonal treatment was initiated on the day following transplantation.

Transplants

Our intention was to remove the three grafts successively

at intervals of two weeks (or earlier if the mouse seemed to sustain), but because of the unpredictable life span of the recipient the interval before removal varied. If only one graft was growing and the mouse seemed to be in good health, we tried to keep the transplant growing as long as possible. Only first generation grafts were used. After extirpation, transplants were fixed in 10% formalin and embedded in paraffin. The whole sample was sectioned and stained with hematoxylin-eosin for histological evaluation according to a standardized schedule.

Blood sampling

Before the mice were sacrificed, blood was drawn by venipuncture from the retro-orbital sinus using heparinized microcapillary tubes. Plasma was separated by centrifugation and stored at -70°C until estradiol- 17β (E_2) was assayed radioimmunologically (46) in all samples simultaneously. In all cases at least 200 μl plasma was obtained.

RESULTS

The endometrium was in a proliferative phase in four patients and in a secretory phase in four. Thirty-two mice were transplanted, but 8 died without providing any histologically evaluable grafts.

The fate of the 96 grafts is shown in Fig. 1. In all, 32 of the grafts removed (33%) had residual epithelial structures. The accepted transplants grew as well defined nodules, encapsulated within a layer of connective tissue in the subcutaneous space, and could easily be extirpated. Three grafts were necrotic. Twenty-one of the grafts with recognizable endometrium were obtained from living mice, while the remaining 11 grafts were taken from mice that had died spontaneously less than 12 hours before. The histological examination revealed that the endometrial structures were still well-preserved. Six evaluable grafts

were obtained from untreated mice, 11 from estrogen-treated mice, 7 from gestagen-treated mice and 8 from danazol-treated mice. The numbers of evaluable samples in the various cycle days, exposed for the different treatment regimens is shown in Tables I-IV. There was no difference in taking rate with regard to cycle phase in the primary tissue.

The life span of the mice was 6-57 days (mean 22 days), and there were no differences in survival between the treatment groups. Mice which were not killed died of a wasting disease.

The ages of the grafts at extirpation is shown in Tables I-IV. The mean age for estrogen-treated grafts was 21 days, for MPA-treated grafts 27 days and for both danazol-treated and untreated grafts 20 days.

Certain observations were common to all heterografts, irrespective of the treatment schedule or of the menstrual phase of the primary tissue. A layer of connective tissue (Fig. 2) surrounding the grafts was seen after 7 days. This fibrosis, which had the form of a capsule-like structure, was most pronounced in the estrogen-treated grafts and thinner and more cellular in the danazol-treated grafts.

Tongues of fibrous tissue extended into the grafts and gradually replaced the specific endometrial stroma (Fig. 3). As long as the ingrowing fibrous tissue was loose and low in collagen, the epithelial height appeared unaffected although the cells showed some degree of polymorphism. With progressive fibrosis, reaching the basal laminae, the glands became narrow and the epithelium flat (Fig. 4); in some glands the nuclei were pyknotic and the epithelial cells had loosened from the surroundings (Fig. 5). However, the fibrotic tissue was never seen to penetrate into the

glands, nor did fibroblasts grow into the lining epithelium from the surface. A progressive degeneration and vanage of the grafts seemed to happen and the grafts were concomittantly replaced by fibrous tissue.

In no case did the grafts have the same histologic appearance as the primary endometrium, not even in the control animals. The epithelial surface was often fringed and budding was frequently noted.

An inflammatory reaction of varying intensity was also seen in the fibrotic tissue surrounding the grafts, sometimes even within the grafts, with varying degrees of hyperemia and infiltration of inflammatory cells.

The various types of injected hormones induced distinct morphological changes in areas where the endometrial stroma was preserved. These effects were independent of the cycle phase of the primary tissue at the time of the transplantation.

Administration of polyestradiol phosphate (Table I) gave rise to the most pronounced effects on the transplants. After 10-12 days the glands were dilated. After 14-15 days, the glandular epithelium had an active proliferative appearance with nuclear stratification (Fig. 6), and after 21 days of treatment mitoses and cilia were found (Fig. 7). Some glands showed an adenomatous hyperplasia. No stromal mitoses were seen and, moreover, decidual change was never found in the stroma.

MPA treatment (Table II) resulted in narrow glands with a low, inactive appearing epithelium when the primary tissue was in a proliferative phase (Fig. 8). When the primary tissue was in a secretory phase, the glands in the grafts were sometimes found to be dilated, probably owing to retained secretion (Fig. 9). No mitotic figures were found, irrespective of the menstrual phase pattern in the primary endometrium. After 15 days of treatment, a deci-

dual reaction had developed in the stroma (Fig. 10) and distinct basal vacuoles were to be seen (Fig. 11).

Heterografts exposed to danazol (Table III) showed glands of varying size and no specific menstrual phase pattern. After only 6 days of treatment, the epithelium was of low to medium height, and the nuclei were large and light (Fig. 12). There were few atrophic glands; the stroma was cellular and there was no decidual change.

In most of the grafts from untreated mice (Table IV) the epithelium was low or medium in height and of inactive appearance, and glands were narrow, only one graft having dilated glands. Already after 10 days, the epithelium had a polymorphic appearance and the nuclei were dark (Fig. 13). In sections from two patients the glandular epithelium had undergone squamous cell metaplasia and comprised small nodules (Fig. 14).

The plasma E_2 concentrations in the different treatment groups are shown in Tables I-IV. With one exception the E_2 concentrations in the estrogen-treated mice were well above those in the other three groups in which, again with one exception (among the MPA-treated mice), they were within the same range.

DISCUSSION

Our study has confirmed that human uterine endometrium can be transplanted into nude mice. The taking rate was 33%, the total loss of operated animals 25%, and the mean survival 22 days after the primary operation. The short life span was probably due to their environment not being germ-free; under conventional conditions, nude mice usually survive for only 2-3 months (16-18,21,26).

The administration of various steroid hormones was found to induce histological changes in the grafts. The morpho-

logical changes found in the grafts after estrogen and MPA-treatment were similar to those seen in normal eutopic human endometrium after corresponding treatment (47,48 a.o.). Although glandular atrophy in the endometrium has been reported after danazol-treatment (49-51, a.o.), none of the endometrial grafts exposed to rather high doses of danazol in our study showed unequivocal signs of atrophy.

On the other hand, a polymorphic appearance of the epithelial cells was noted, chiefly in the control group, irrespective of the histological menstrual phase in the eutopic endometrium. The non hormone-specific alterations observed in our grafts may have been the result of the biochemically abnormal milieu in the recipient, and perhaps to the impediment of graft nutrition by surrounding connective tissue. There was a striking similarity between the histological patterns found here and those of endometriotic structures found in humans (52). This may suggest that when endometrial tissue is transferred to an abnormal milieu and surrounded by ingrowing fibrous tissue, it assumes morphological features which are very similar to those of endometriotic tissue.

In nude mice, the gonadal function is defective, puberty is considerably delayed, and already after six weeks of age their estradiol-17 β concentrations are much lower than in normal (haired) mice (53,54). Although they begin to ovulate at the age of 2.5 months, owing to a defective endogenous gonadotropic function, follicular growth is retarded and the oocytes degenerate rapidly; after the age of 4 months no further ovulation occurs (55-59).

The mice used in our study were 6-11 weeks old at the primary operation, and can thus be assumed to have had low E₂ and progesterone concentrations. The mean E₂ concentrations in mice treated with MPA or danazol were not significantly lower than those in the control mice, whose

concentrations agreed well with those previously found in anovular mice (53). These findings indicate that most mice were anovulatory.

The effects of hormone administration were most pronounced after more than two weeks of treatment. The main reason for extirpating grafts before two weeks of growth was the poor condition of the mouse, which may well have affected graft nutrition causing defective hormonal response. Four of the 7 grafts less than 14 days old were taken from dead mice, and the remaining 3 grafts from 2 living mice; there was no difference in hormonal effect, however, between grafts obtained from dead mice and those from living mice. Delayed take of up to several weeks, has been reported by several workers (21,24,26,27,34,39); this delay may be partly due to the time required for vessels to grow into the graft and provide optimal nutrition. Revascularization has been found in human skin grafts after 6-10 days (42). After this period the hormonal effect should have become obvious as well.

Surrounding and ingrowing fibrotic tissue was found in most grafts. Most types of grafted tissue have been reported to be well-defined (22,23,27,35,37,39), being circumscribed by a layer of connective tissue (26). In our study, fibrosis was most pronounced in estrogen-treated mice, whereas in the danazol-treated mice it was less extensive, more cellular and low in collagen. We found no actual destruction of the epithelial structures by fibrous tissue from the host, nor any fibroblasts growing into the lining epithelium from the surface. When no specific stroma was left around the glands, however, the epithelium was found to have atrophied. This suggests that the preservation of a specific endometrial stroma is necessary for maintaining normal morphology of the glandular epithelium. A similar observation in a clinical material was reported by Cullen (60). Morphological changes in the

glandular epithelium resulting from a given hormone treatment were only found in those areas where the specific stroma was preserved. This suggests that an intact specific stroma is also necessary to enable the glandular epithelium to respond to hormonal substances present in the circulation.

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TABLE I. Menstrual cycle day at the time of implantation, determined histologically according to Noyes (45), age of the graft in the mouse, E₂ concentrations in the mouse plasma when the graft was extirpated and histological picture of human endometrial grafts in nude mice exposed to polyestradiol phosphate treatment.

Pat. no.	Cycle day	Age of the graft (days)	E ₂ /plasma pmol/l	Alive or dead	Histology
5	14	7	ND	dead	dilated glands, medium high epithelium, no specific phase pattern
6	10	10	1320	alive	cystic dilated glands, low epithelium
4	18	14	240	alive	dilated glands, low epithelium
2	20	15	1600	alive	some narrow, some dilated glands; medium high epithelium; pseudo-stratified nuclei
1	14	17	4140	alive	some narrow, some dilated glands; medium high epithelium, no specific phase pattern*
3	17	20	1060	alive	narrow glands, low epithelium*
8	22	21	9200	alive	dilated glands, medium high epithelium, pseudo-stratified nuclei, mitoses
7	11	22	ND	dead	dilated glands, high polymorphic epithelium
2	20	30	1540	alive	dilated glands, high epithelium, pseudo-stratified nuclei, mitoses
1	14	37	1140	alive	dilated glands, high foliated epithelium, pseudo-stratified nuclei, mitoses
1	14	40	ND	dead	dilated glands, high epithelium, pseudo-stratified nucleic, mitoses

* = no specific stroma surrounded the glands

ND = not determined

TABLE II. Menstrual cycle day at the time of implantation, determined histologically according to Noyes (45), age of the graft in the mouse, E_2 concentrations in the mouse plasma when the graft was extirpated and histological picture of human endometrial grafts in nude mice exposed to MPA-treatment.

Pat. no.	Cycle day	Age of graft (days)	E_2 /plasma pmol/l	Alive or dead	Histology
6	10	9	ND	dead	narrow glands, atrophic epithelium, no decidua
2	20	15	260	alive	some narrow, some dilated glands, high epithelium, partly pseudo-stratified nuclei; vacuoles basally and apically, decidua, stromal edema
3	17	20	160	alive	dilated glands, low epithelium, decidua
7	11	28	900	alive	narrow glands, atrophic epithelium, decidua
3	17	30	ND	dead	dilated glands, low epithelium, decidua
2	20	30	120	alive	dilated glands, low epithelium, pronounced decidual reaction
1	14	57	80	alive	narrow glands, atrophic epithelium, decidua

ND = not determined

TABLE III. Menstrual cycle day at the time of implantation, determined histologically according to Noyes (45), age of the graft in the mouse, E_2 concentrations in the mouse plasma when the graft was extirpated and histological picture of human endometrial grafts in nude mice exposed to danazol treatment.

Pat. no.	Cycle day	Age of graft (days)	E_2 -plasma pmol/l	Alive or dead	Histology
4	18	6	ND	dead	medium high epithelium
6	10	10	ND	dead	some narrow, some unregularly dilated glands, varying epithelial height
2	20	15	200	alive	dilated glands, medium high epithelium, stromal edema
3	17	20	120	alive	dilated glands, low-medium high epithelium
5	14	24	ND	dead	dilated glands, medium high epithelium, pseudostratified nuclei
7	11	28	240	alive	narrow glands, one cystic dilated gland, low-medium high epithelium, atrophy in the narrow glands, stromal edema
3	17	29	ND	dead	some narrow, some cystic dilated glands, medium high epithelium
2	20	30	120	alive	dilated glands, low epithelium

ND = not determined

TABLE IV. Menstrual cycle day at the time of implantation, determined histologically according to Noyes (45), age of the graft in the mouse, E₂ concentrations in the mouse plasma when the graft was extirpated and histological picture of human endometrial grafts in nude mice not exposed to any hormonal treatment.

Pat. no.	Cycle day	Age of graft (days)	E ₂ -plasma pmol/l	Alive or dead	Histology
6	10	10	140	alive	narrow glands, low-medium high epithelium, squamous cell metaplasia
6	10	16	ND	dead	narrow glands, medium high epithelium, partial atrophy
1	14	17	160	alive	some narrow, some dilated glands, low-medium high epithelium, squamous cell metaplasia
8	22	21	360	alive	dilated glands, medium high epithelium, partial atrophy
3	17	28	180	alive	narrow glands, low epithelium
2	20	29	ND	dead	narrow glands, low-medium high epithelium

ND = not determined

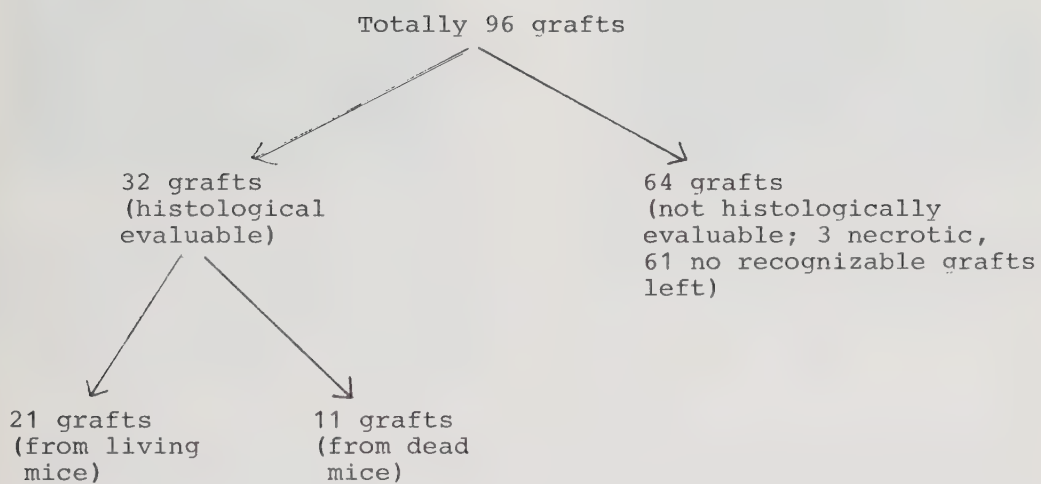


Fig. 1 The fate of 96 grafts of human normal uterine endometrium transplanted s.c. into nude mice.



Fig. 2 Human endometrium transplanted s.c. into a nude mouse. A surrounding fibrous capsule is seen. After 21 days without any treatment. x 100

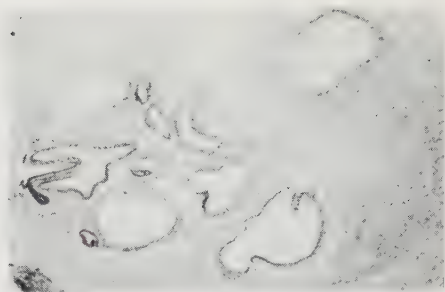


Fig. 3 Human endometrium transplanted s.c. into a nude mouse. Tongues of fibrous tissue extend into the graft and gradually replace the specific endometrial stroma. After 40 days of polyestradiol phosphate treatment. x 100

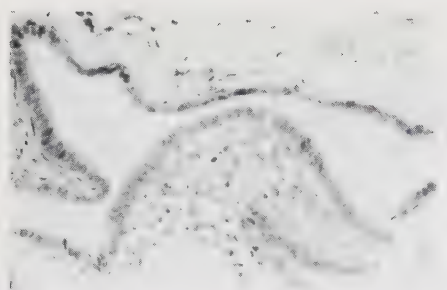


Fig. 4 Human endometrium transplanted s.c. into a nude mouse. In areas where the specific stroma is preserved, the epithelial height is unaffected, but where the fibrosis has reached the glands, the epithelium has become flat. After 30 days of polyestradiol phosphate treatment. x 400

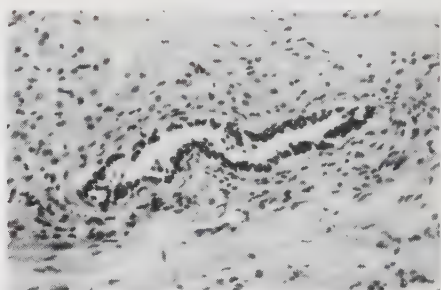


Fig. 5 Human endometrium transplanted s.c. into a nude mouse. The surrounding fibrosis has replaced all specific endometrial stroma, the gland has become narrow, the epithelium low with pyknotic nuclei which partly has loosened from the surroundings. After 9 days of MPA treatment. x 400

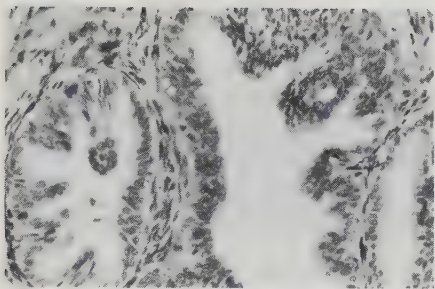


Fig. 6 Human endometrium transplanted s.c. into a nude mouse. The glandular epithelium is foliated and the nuclei stratified. After 37 days of polyestradiol phosphate treatment. x 400

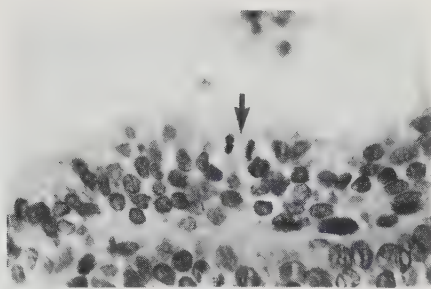


Fig. 7 Human endometrium transplanted s.c. into a nude mouse. Mitoses are found. After 37 days of polyestradiol phosphate treatment. x 1000

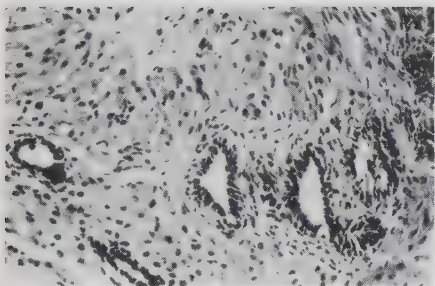


Fig. 8 Human proliferative endometrium transplanted s.c. into a nude mouse. The glands are narrow, inactive appearing with low epithelium. After 28 days of MPA treatment. x 400

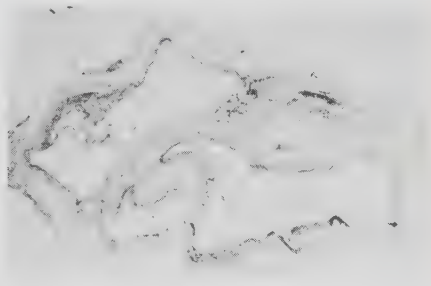


Fig. 9 Human secretory endometrium transplanted s.c. into a nude mouse. The glands are dilated with flat epithelium. After 30 days of MPA treatment. x 100



Fig. 10 Human endometrium transplanted s.c. into a nude mouse. A pronounced decidual reaction is seen. After 30 days of MPA treatment. x 100

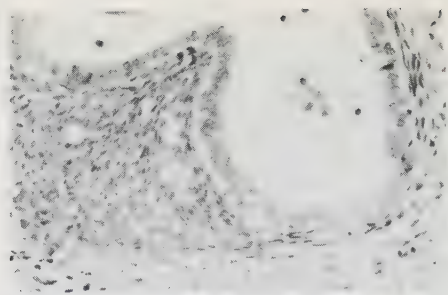


Fig. 11 Human endometrium transplanted s.c. into a nude mouse. Distinct basal vacuoles are seen. After 15 days of MPA treatment. x 400

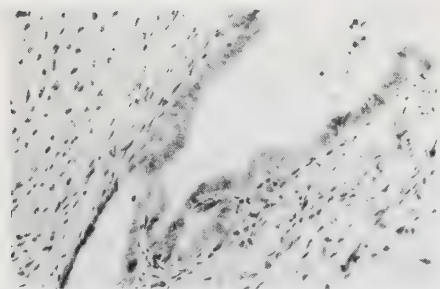


Fig. 12 Human endometrium transplanted s.c. into a nude mouse. The epithelium is medium high and the nuclei large and light. After 10 days of danazol treatment. x 400

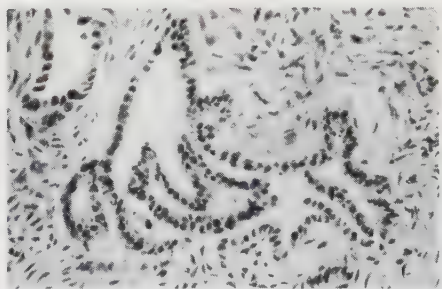


Fig. 13 Human endometrium transplanted s.c. into a nude mouse. The epithelium has a polymorphic appearance and the nuclei are dark. After 21 days without any hormonal treatment. x 400

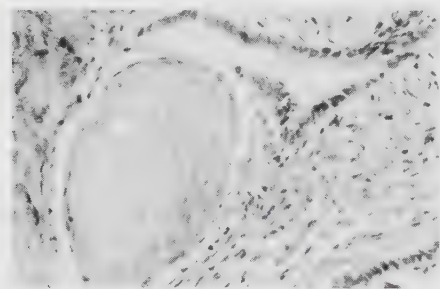


Fig. 14 Human endometrium transplanted s.c. into a nude mouse. A squamous cell metaplasia is found in some glands. After 16 days without any hormonal treatment. x 400

HUMAN UTERINE ENDOMETRIUM AND ENDOMETRIOTIC TISSUE TRANS-
PLANTED INTO NUDE MICE. Morphological effects of various
steroid hormones.

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Running heading: Endometrium and endometriotic tissue
transplanted into nude mice.

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ABSTRACT

In a study of the morphological effects of various steroid hormones on human uterine endometrium and endometriotic tissue, specimens from 6 women were transplanted subcutaneously into 24 nude mice. In each case, specimens collected simultaneously from the woman were transplanted to four mice, uterine endometrium to one lateral abdominal wall and endometriotic tissue to the other.

All 24 mice were given polyestradiol phosphate subcutaneously the first day after operation. After a two week interval, of each 4 mice, one was given polyestradiol phosphate, one medroxyprogesterone acetate, one danazol, and one received no further injections. This treatment continued for 8 weeks, after which the mice were killed and the grafts extirpated.

Histological changes in the grafts varied according to the treatment schedule, but were very similar in both types of tissue. Our findings suggest that histological differences between uterine endometrium and endometriotic tissue seen under natural conditions may, at least partly, be due to variations in environmental factors.

INTRODUCTION

Autografting of uterine endometrium to the peritoneal cavity of various laboratory animals is the method that has been most widely used in studying ectopic endometrium in vivo (1,2,3,4,5). In vivo studies of human ectopic endometrium and endometriotic tissue have hitherto been hampered by adverse immunological reactions.

The mouse mutant "nude", has been found suitable as a host for heterografts, owing to its congenital thymus aplasia (6) resulting in a defective T-lymphocyte system (7), and its low serum immunoglobulin concentrations (7,8). Several studies have shown that human tissue transplanted into nude mice generally retains its histological, chromosomal and biochemical properties (9).

Different types of normal and tumorous human tissue have been transplanted into nude mice (9), and the susceptibility of the tissue to various pharmacological substances has been found to be preserved after transplantation (10,11,12,13,14).

In a previous study we successfully transplanted normal human endometrium into nude mice (Bergqvist et al., submitted 1984) and Zamah et al (15) recently published a study of human endometrium and endometriotic tissue transplanted into nude mice. However, no experimental study has previously been reported on human endometrium and endometriotic tissue obtained simultaneously from the same woman and placed into the same milieu.

The aim of this study was to investigate the effect of various steroid hormones on the morphology of human endometrium and endometriotic tissue, obtained simultaneously from the same woman, after transplantation into nude mice.

MATERIAL AND METHODS

Nude mice (nu/nu -BALB/c/AB spf. qq) were obtained from Dr. C.W. Friis, Laboratory Animals Breeding and Research Centre, Gl. Bomholtgaard, Ry, Denmark. The animals were kept in open polypropylene cages covered by a stainless steel lid. The cages were kept in a laminar air flow bench (ASSAB Medicin AB, Sundbyberg, Sweden) at 26-28°C and 10 hours light per diem. The bedding consisted of aspen tree granulate (Torrax, ALAB Laboratorietjänst AB, Sollentuna, Sweden), pre-sterilized by autoclaving at 120°C for one hour. The cages were changed weekly and cleaned manually. The diet consisted of pellets with mineral and vitamin additives (R3, Ewos-Anticimex, Södertälje, Sweden), pre-sterilized by autoclaving at 120°C for one hour. Sterile water supplemented with HCl to pH 2.5-2.8 was supplied *ad libitum* by means of a glass bottle with a conical stainless steel top and changed 3 times a week. All other items of equipment were changed once weekly. The animals were handled aseptically. The mice were 8-14 weeks old at the primary operation.

Human endometriotic tissue was obtained from 6 women (aged 26 - 36 years) at laparotomy. Small endometriotic lesions were macroscopically freed from surrounding connective tissue by knife. Endometriotic tissue covering the inside of endometriotic cysts was freed of encapsulating fibrous tissue by gentle scraping with a knife. Uterine endometrium was obtained simultaneously by curettage. The tissue samples were handled aseptically and kept supplied with minerals and vitamins by being immersed immediately after collection in sterile tubes containing Parkers medium 199 (SBL, Stockholm, Sweden).

One piece of the tissue sample was fixed in 10% formalin, dehydrated and imbedded in paraffin. Sections, 6 µm in thickness, were stained with hematoxylin-eosin for histo-

logical identification of the structures. The menstrual cycle phase in the uterine endometrium was determined essentially according to Noyes et al. (16). The remaining part of the tissue samples was transplanted into nude mice.

The grafting procedure was performed aseptically in a laminar air flow bench within 60 min. after the tissue collection in the operating theater. The mice were anesthetized with cloralhydrat (20 mg/ml) 0.5 ml given intraperitoneally.

The larger tissue pieces were cut with a safety-razor blade into smaller pieces measuring about 2-3 mm³. The cutis within the operation area was disinfected by means of 0.5% chlorhexidine suspension. A cutaneous incision about 5 mm in length, was made in the lateral abdominal wall of the mouse and subcutaneous debridation was performed with a pair of blunt-ended scissors. Two pieces of endometriotic tissue were implanted subcutaneously (s.c.) in one flank, one in the frontal region, the other in the hinder region. The uterine endometrium was transplanted to the other flank in a similar manner. The cutis was sutured with one stitch of Suturamide on each side.

Tissue samples from each woman were transplanted to four mice. Only first generation grafts were studied. Since a previous study (Bergqvist et al., in manuscript) showed a somewhat higher taking rate of human endometrium transplanted into nude mice after estrogen administration, the first day after the primary operation all 24 mice were given one injection of 0.02 mg (0.1 ml) polyestradiol phosphate (Estradurin^R 80 mg/ml, diluted with saline) s.c. Starting two weeks after operation, of each group of four mice, one received no further injections and served as a control, the other three being injected s.c., one with polyestradiol phosphate (Estradurin^R 80 mg/ml, diluted

with saline) 0.02 mg (0.001 mg/g b.w.) 0.1 ml once every two weeks; one with medroxyprogesterone acetate (MPA, Depo-Provera^R 50 mg/ml) 5 mg (0.25 mg/g b.w.) 0.1 ml once weekly; and one with danazol 4 mg (0.2 mg/g b.w.) dissolved in 0.1 ml of a sterile mixture of 44% peanut oil, 46% benzyl benzoate and 10% phenyl carbinol once daily. Only one mouse died spontaneously before the end of the observation period. After eight weeks of differentiated treatment, the surviving 23 mice were killed and all remaining transplants were extirpated, fixed in 10% formalin, dehydrated and imbedded in paraffin. The whole grafts were sectioned and stained with hematoxylin-eosin for histological evaluation.

RESULTS

Two of the endometria were in the proliferative phase and 4 in the secretory phase. The site of the endometriotic lesions and the menstrual cycle phase of the endometrium are shown in Table I. A total of 96 grafts were inserted, 33 of which had survived disintegration at the end of 10 weeks and were extirpated for examination. Epithelial structures could be identified in 28 grafts (29%): 2 estrogen-treated, 2 gestagen-treated, 3 danazol-treated and 4 untreated endometriotic grafts; and 5 estrogen-treated, 4 gestagen-treated, 4 danazol-treated and 4 untreated endometrial grafts.

The taking rate was 50% higher for endometrium than for endometriotic tissue. The histological appearance of the evaluable grafts are given in Table II.

All grafts were surrounded by a capsule of fibrous tissue, and the fibrosis extended into the grafts to varying degrees. The fibrosis was very thick and collagen rich in the estrogen-treated grafts but less compact in the danazol-treated grafts. In this respect there was no

difference between endometrial and endometriotic grafts. The grafts seemed to be resolved gradually and replaced by fibrous tissue. In the fibrotic area near the remaining endometrial or endometriotic structures, small remnants of glands in different stages of degeneration were found without any residual surrounding specific stroma (Fig. 1).

In no case was the graft histologically identical to the original tissue. The histological appearance of the endometrial grafts was similar to that found in a previous study on human endometrium transplanted into nude mice (Bergqvist et al. in manuscript). The histological changes in the endometriotic grafts closely resembled those found in the endometrial grafts (Figs. 2-5), i.e., proliferative epithelium was observed in the estrogen-treated grafts and an inactive pattern in the gestagen-treated and danazol-treated grafts. Moreover the untreated grafts of the two tissue types took on the same histological appearance after transplantation to the same host milieu. There were hemosiderin-loaded macrophages in both types of tissue. A slight inflammatory reaction with infiltration of plasma cells, lymphocytes, leukocytes, macrophages and occasional foreign body giant cells was found within the grafts and in surrounding fibrous tissue. Cholesterol crystals were sometimes found. The grafts were well preserved in their central parts without signs of fresh bleeding.

DISCUSSION

This experimental model made it possible to study human endometrium and endometriotic tissue derived simultaneously from the same woman and transplanted into a similar host milieu. After 10 weeks, no difference was found in the histological appearance of the two types of tissue in untreated mice. Moreover, the histological changes induced by hormonal treatment were similar in the two tissue types. Even though the endometriotic grafts contained

somewhat fewer glands than did the endometrial grafts, the amount of epithelial structures was sufficient to permit comparison between the two tissue types.

Both the inflammatory reaction and the fibrosis, and the histological changes induced by estrogen treatment, were very similar to those reported recently by Zamah et al. (15).

To sum up, this study confirms that human endometrium and endometriotic tissue can be transplanted into nude mice where they take on a similar histological appearance and respond in a similar manner to steroidal influences. These findings suggest that the histological discrepancies seen under natural conditions between human uterine endometrium and endometriotic lesions may, at least in part, be the result of environmental differences.

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TABLE I

Histologically determined menstrual phase in the uterine endometrium and localization of the endometriotic lesions in 6 women.

Pat no	Cycle day in uterine endometrium	Localization of endometriotic lesions
1	12	endometrioma*
2	17	endometriomas* and a few small ovarian lesions
3	18	endometrioma* and a few small ovarian lesions
4	18	endometrioma*
5	21	endometrioma*
6	22	endometrioma* and a few small peritoneal lesions

*) ovarian cyst larger than 3 cm in diameter

TABLE II.

Histological pattern in human endometrium and endometriotic tissue obtained simultaneously from 6 women and transplanted into 24 nude mice, 4 nudes per woman (endometrium to one, endometriotic tissue to the other side of the same mouse). All mice obtained an initial injection of polyestradiol phosphate. Two weeks later, an 8 week treatment period was introduced in 3 of them giving polyestradiol phosphate, MPA and danazol respectively. One mouse received no further treatment.

	Polyestradiol phosphate			MPA		Danazol		Control	
	endometrium n=4	endometriotic tissue n=2	endometrium n=4	endometrium n=2	endometriotic tissue n=2	endometrium n=4	endometriotic tissue n=3	endometrium n=4	endometriotic tissue n=4
Glands	several, varied-sized, mostly round	few, dilated, varying forms	several, varying sizes and forms	several, varying sizes and forms	several, varying sizes and forms	several, varying sizes and forms	several, varying sizes and forms	several, varying sizes and forms	several, varying sizes and forms
Epi- thelium	high - medium- high, proliferative pattern, stratified oval nuclei of similar size, mitoses, cilia	high - medium- high, foliated, proliferative pattern, stratified, pale oval nuclei of similar size, cilia	low-medium high, vacuoles, partly atrophic partly atypical	medium-high, vacuoles, no atrophy, atypical	low - medium-high, partly strati- fied nuclei, no atrophy, partly atypical	low - medium-high, partly strati- fied oval nuclei, in- active pattern, atypical	low-medium high, partly atro- phic, partly atypi- cal	high - medium-high, partly strati- fied oval nuclei, in- active pattern, atypical	high - medium-high, partly strati- fied nuclei, mitoses, villi, partly atypical
Stroma	cellular	cellular	decidua, sometimes edema	pronounced decidua	no decidua,	no decidua,	no decidua	only few remaining cells	cellular dark nuclei

n = number of mice

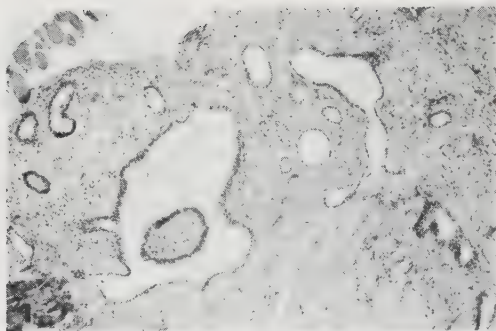


Fig. 1 Human endometrium 10 weeks after transplantation s.c. into a nude mouse. No hormonal treatment except one initial injection of polyestradiol phosphate. Fibrotic tissue has grown towards the central part of the graft leading to degradation of glands deprived of surrounding specific stroma. x 100

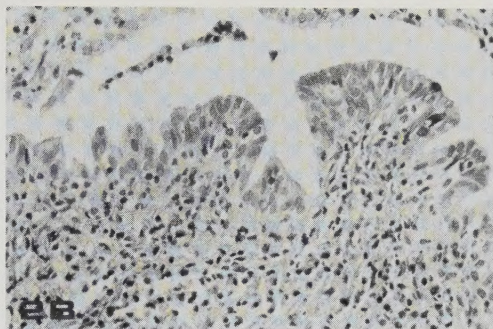
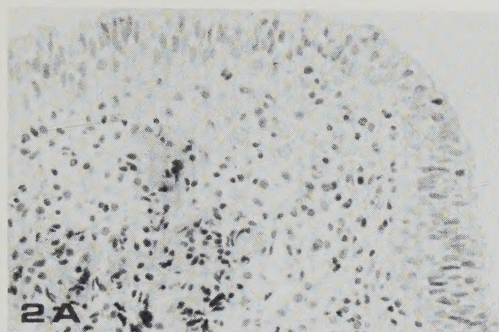


Fig. 2a Human endometrium and b) endometriotic tissue transplanted s.c. into a nude mouse and treated with polyestradiol phosphate for 10 weeks. The epithelium is high and the nuclei ovale and stratified. x 400

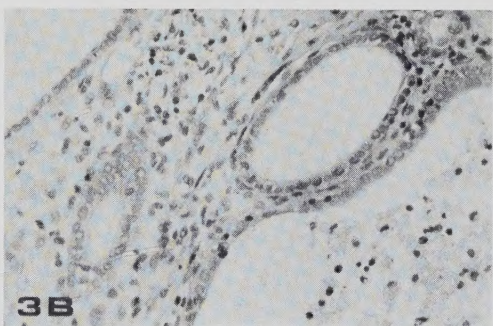
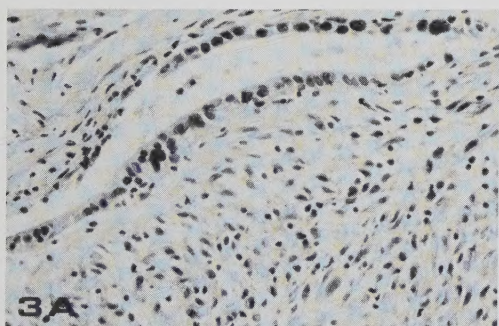


Fig. 3a Human endometrium and b) endometriotic tissue transplanted s.c. into a nude mouse and treated with MPA for 8 weeks starting 2 weeks after one initial injection of polyestradiol phosphate. The epithelium is low or medium-high, partly atypical and partly inactive. A decidual reaction is seen in the endometriotic graft. x 400

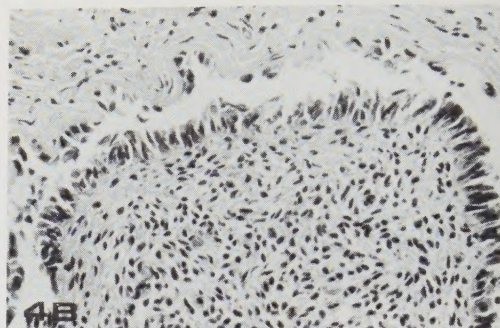
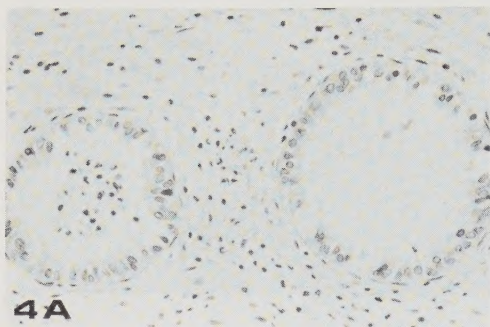


Fig. 4a Human endometrium and b) endometriotic tissue transplanted s.c. into a nude mouse and treated with danazol for 8 weeks starting 2 weeks after one initial injection of polyestradiol phosphate. The epithelium is medium-high and partly atypical. x 400

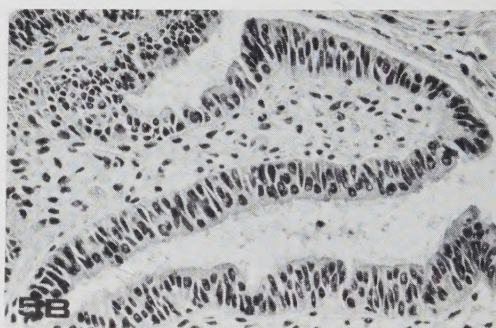
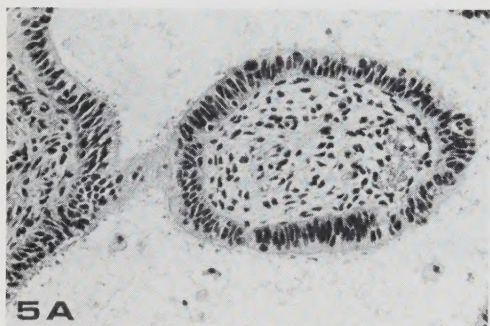


Fig. 5a Human endometrium and b) endometriotic tissue 10 weeks after transplantation s.c. into a nude mouse without hormonal treatment except for one initial injection of polyestradiol phosphate. The epithelium is high and the nuclei are stratified. x 400

btj datafilm, Lund 1985